SUPER-SUPPRESSION IN HAPLOID AND DIPLOID YEAST

by

Janina F. Sliwowska

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Department of Genetics,
University of Edinburgh

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SUMMARY

Suppression of $ad_{2.1}$ haploids and homozygous diploids was observed to be far less effective than that of 4 other ochre nonsense alleles ($lys_{1.1}$, $arg_{4.17}$, $his_{5.2}$, $try_{5.48}$). The penetrance of the suppressed phenotype on adenineless medium (-AD) varied considerably and showed an approximate correlation with suppressor genotype. It was demonstrated that expression of suppressors on -AD plates could be considerably increased by a continual selection regimen. Loss of this improved growth ability by the growth of suppressed strains in a medium containing adenine, or by changes in their ploidy, suggested that increased suppressor expression resulted from physiological adaptation rather than from genetic change.

Growth patterns in liquid -AD were used to study some characteristics of the adaptation mechanism. Duration of the lag phase appeared to be correlated with initial ability to grow on -AD plates, and thus with the suppressor genotype. The slope of the exponential portion of the -AD growth curve was usually very similar to that in a comparable medium containing adenine (+ALL), suggesting that those cells capable of growth did so at the same rate in -AD as when adenine was not a limiting factor. However, although reinoculation of cultures grown in -AD into fresh -AD reduced the lag phase considerably, this phase was not usually eliminated; apparently, adaptation effects were easily lost.

The possibility that growth was attributable to selection of one or a small number of mutant cells was discounted on the grounds

that not only did the -AD pregrowth effect of shortened lag period disappear after growth of such cultures in complete medium prior to reinoculation into fresh -AD, but also the length of the lag found in the first -AD culture was often such that any hypothesis based on selection of mutant cells would also require to invoke an adaptation of such mutants.

Prior selection on -AD plates decreased the lag period in -AD liquid (or enabled growth to occur where none had before). It was deduced that at least some aspects of adaptation were common to responses to both selection media.

Selection experiments using suppressed isolates of strain 2885-32B, which contained a different set of nonsense alleles ($ad_{5,7-101}$, $lys_{1.1}$, $leu_{2.1}$, $ura_{4.1}$, $try_{1.1}$, $met_{8.1}$), revealed cases where selection for increased growth on one omission medium resulted in correlated growth responses on other omission media. The presence of additional suppressors was ruled out. Cytoplasmic changes affecting suppressor activity were invoked to explain these results.

Suppressed $ad_{2.1}$ $ura_{4.1}$ isolates similarly showed correlated responses to selection. Such responses were explicable in terms of modification either of interelated biochemical pathways or of the suppression mechanism.

Possible models for the mechanism of adaptation were discussed. Assuming modification of suppressor efficiency underlies adaptive responses, such selection experiments were considered potentially useful in understanding various aspects of the suppression mechanism not readily analysed by classical genetic methodology.

CONTENTS

CHAPTER I

INTRODUCTION

Over a decade has passed since the elucidation of the triplet nature of the genetic code by Crick et al. (1961) and the subsequent recognition of the amber (UAG), and ochre (UAA) and umber (UGA) codons as the polypeptide chain terminating signals during mRNA translation. Since then, mutations to these so-called "nonsense" codons and the modification of their expression by further mutations at other loci have been widely studied using both biochemical and genetic techniques.

It is now well established that the expression of any particular mutation can be influenced by any one of the many factors involved in the protein synthesizing system. Of particular interest are the cases in which the fidelity with which the genetic code is translated is altered. Those mutations whose gene products alter the translation machinery in such a way that certain other mutations are no longer expressed are known as "informational suppressors".

The existence of informational suppressors was first postulated by Yanofsky and St. Lawrence (1960). The most frequent, and therefore the most thoroughly investigated, form of informational suppression occurs via altered t-RNA species, which appear able to suppress nonsense, missense or even frameshift mutations. Perhaps the most interesting case of informational suppression, and indeed the most useful in providing a tool for genetic analysis, is that of nonsense suppression.

Nonsense suppression in procaryotes was first studied by Benzer and Champe (1961, 1962) and Garen and Siddiqi (1962). Since then extensive biochemical and genetic studies, mainly using E. coli and its phages, have led to the elucidation of the molecular basis of the mechanism of nonsense suppression in procaryotes.

The only eucaryotic organism in which informational suppression has been studied in depth is Saccharomyces cerevisiae. The study of nonsense suppression in yeast began in 1963 with the discovery of "super-suppressors" (Hawthorne and Mortimer, 1963), now known to be analogous to the nonsense suppressors of bacteria. The model of nonsense suppression proposed for the procaryotic system has served as a framework upon which the results of subsequent investigations in yeast have been interpreted. The similarity between nonsense suppression in bacteria and in yeast is extensive. However, details of the two systems differ.

Informational suppression in procaryotes has been extensively reviewed (Gorini and Beckwith, 1966; Garen, 1968; Gorini, 1970). However, since the mechanism of super-suppression in yeast has been inferred largely from the situation in E. coli, it may be useful to give a brief résumé of the procaryote model of nonsense suppression before dealing with the eucaryote situation. Details and further references may be found in the above reviews.

The three nonsense codons UAG (amber), UAA (ochre) and UGA (umber) usually occur only as "punctuation" signals between regions of amino-acid coding (Nicols, 1970; Lu and Rich, 1971), but they can also arise via mutation of a sense triplet (Brenner et al., 1965). Nonsense codons have no cognate aminoacyl-tRNA species (Nirenberg

et al., 1965; Soll et al., 1965). When they occur in the mRNA of an unsuppressed strain, polypeptide chain elongation terminates at the site of the nonsense codon (Stretton and Brenner, 1965). If the nonsense codon occurs in an intra-cistronic region of mRNA, polypeptide fragments are formed (Sarabhai et al., 1964; Englehardt et al., 1965; Zinder et al., 1966; Suzuki and Garen, 1969; Model et al., 1969). A mutant phenotype usually results. In the presence of a nonsense suppressor, Su^+, completed proteins are produced due to insertion of an amino-acid at the nonsense codon (Notani et al., 1965; Kaplan et al., 1965; Weigert and Garen, 1965; Weigert et al., 1965, 1967; Chan and Garen, 1969, 1970). Completed proteins and peptide fragments are thus produced in a ratio dependent on the efficiency of the suppressor i.e. frequency of $\frac{\text{translation}}{\text{translation + termination}}$ at the nonsense codon. Suppression appears to be most frequently mediated by a tRNA whose anticodon can recognise and translate the nonsense codon. Such recognition can be accounted for, in several cases, by mutation of a single base pair in the anticodon region of the tRNA (Goodman et al., 1968; Altman et al., 1971; Yaniv et al., 1974). However, in some cases, recent evidence has implicated base changes at sites in the tRNA molecule other than the anticodon as conferring the suppressing ability (Hirsh, 1970, 1971; Hirsh and Gold, 1971). From these studies it is concluded that codon-anticodon pairing specificities can be influenced by sites elsewhere in the tRNA molecule. Other investigations have suggested that incomplete enzymic modification of the tRNA molecule leads to recessive suppressor activity (cited in Hartman and Roth, 1973, P.21), in contrast to the dominance exhibited by most suppressors (Capecchi and Gussin, 1965; Signer et al., 1965).

Restoration of protein function depends upon both the position of the nonsense codon in the gene and the functional compatibility of the particular amino-acid inserted by a given suppressor. This is reflected in the characteristic allele - specificity, locus non-specificity, properties of nonsense suppressors, which enables them to be classified according to the alleles which they suppress (Osborn et al., 1967; Krieg and Stent, 1968).

The above model of nonsense suppression predicts that the amino-acids most frequently inserted by nonsense suppressors will be those whose anticodons differ by one base from a nonsense anticodon. Seven amino-acids have anticodons differing by a single base from CUA (the anticodon of amber (UAG) suppressors), while only six are similarly related to UUA (the anticodon of ochre suppressors which, in bacteria, translate both UAA and UAG codons due to 'wobble' in the third position on the codon (Crick, 1966)). No exceptions to these expected amino-acid substitutions have yet been found, a fact which supports the proposed model of nonsense suppression.

Mutation at a tRNA locus to give a nonsense suppressor may have detrimental consequences due either to the removal of wild-type function of the original tRNA species or to excessive reading of normal inter-cistronic terminator codons. The latter possibility may limit the efficiency of suppressors recovered, and may account for some of the pleiotropic effects, notably on growth rates, of some suppressors, particularly of the ochre variety (Gartner and Orias, 1965; Ohlsson et al., 1968; Tevethia et al., 1974). Such effects have implicated the ochre codon as being of particular significance in natural chain termination.

If a tRNA species is coded for by a single, unduplicated cistron, then its mutation to a suppressor, involving loss of the original coding specificity, will mean that the cell lacks a complete decoding capacity, and the mutation will be lethal. Such lethal mutations of indispensable tRNA species can only be maintained in merodiploids containing the wild-type allele (Soll and Berg, 1969a,b; Miller and Roth, 1971).

The ability to suppress nonsense codons without concomitant loss of a specific decoding ability (or any other function) of the original tRNA species is possible only in those situations where additional genes code for equivalent tRNA species. Duplication of the original cistron or the existence of iso-accepting subspecies of the tRNA functionally equivalent to the original species, provide such a situation. Nonsense suppressor mutations are usually recovered in the structural genes of such dispensable tRNA subspecies (Smith *et al.*, 1966; Andoh and Garen, 1967; Goodman *et al.*, 1968; Soll, 1968; Gopinathan and Garen, 1970).

Many lines of evidence support the comparability of super-suppression in *Saccharomyces cerevisiae* and nonsense suppression in *E. coli* and its phages. Since super-suppression in yeast has been recently reviewed (Hawthorne and Leupold, 1974), only a brief survey of this field will be given below.

Super-suppressible alleles have many of the characteristics predicted for nonsense mutants:-

(i) They are not leaky, temperature-sensitive or osmotically-remedial (Hawthorne and Friis, 1964).

(ii) They do not usually exhibit intra-cistronic complementation,

but the few that do show a polarized complementation pattern (Fink, 1966; Manney, 1964).

(iii) They cause chain-termination (Manney, 1968)

(iv) Amber (Hawthorne, 1969a; Stewart and Sherman, 1972, 1973), ochre (Hawthorne, 1969a; Gilmore *et al.*, 1968; Stewart *et al.*, 1972) and UGA (cited in Hartman and Roth, 1973, P.24) codons have been identified at the mutant site of several super-suppressible alleles.

Similarly, much evidence suggests that yeast super-suppressors are analogous to *E. coli* nonsense suppressors:-

(i) Super-suppressors are allele-specific and locus non-specific (Hawthorne and Mortimer, 1963) as are nonsense suppressors (Krieg and Stent, 1968; Osborn *et al.*, 1967). They suppress approximately 30% of mutant sites (Hawthorne and Mortimer, 1963; Manney, 1964), a frequency comparable to that of nonsense suppressors in *E. coli* (Witkin, 1963). This provides further evidence for the identity of pro- and eucaryotic super-suppressible alleles.

(ii) Super-suppressors are dominant (von Borstel *et al.*, 1966; Magni and Puglisi, 1966), as are *E. coli* nonsense suppressors (Signer *et al.*, 1965; Capecchi and Gussin, 1965).

(iii) Super-suppressor loci are thought to encode tRNA genes. Evidence for this is at present mainly inferential (Gilmore and Mortimer, 1966; Magni and Puglisi, 1966; Magni *et al.*, 1966; Bruenn and Jacobson, 1972).

(iv) Data on the mutagenic induction of various classes of suppressor are compatible with the assumption that single base substitutions in the anticodon of specific tRNA species give rise to suppressor activity (Hawthorne, 1969b).

Thus it appears that the fundamental features of super-suppression in this eucaryotic system parallel those of procaryotic nonsense suppression.

However, the yeast system differs in three major respects from that in bacteria: in the specificity of the suppressors, in the number of suppressor sites and in the susceptibility of the suppressor phenotype to modification. Differences in all three aspects are predictable upon consideration of the organization of eucaryotic and procaryotic nuclear and cytoplasmic systems.

Transfer-RNA-mediated nonsense suppressors in E. coli fall into three classes of codon specificity: UAG-specific, UAA-UAG-specific and UGA-specific. Of these classes, only the UAG-specific and UGA-specific are also represented in yeast. UAA-specific and UAA-UAG-specific categories have been distinguished in yeast but the latter class also appears able to suppress UGA, and these "omnipotent" suppressors have recently been proposed to exert their effects via components of the translation mechanism other than tRNA (Hawthorne and Leupold, 1974). The lack of suppressors analogous to bacterial ochre suppressors and the presence of ochre-specific suppressors therefore distinguish yeast super-suppression.

Why super-suppressors with a UUA anticodon able to recognise both UAA and UAG are not recovered remains unexplained. Ochre-specific suppressors may contain a modified base in the first position of the anticodon, permitting recognition of A but not G in the third position of the codon. One candidate for such a base is inosine (I), which arises by deamination of A. It was predicted (Bock, 1967) that if A in the first position of the anticodon AUA (derived by mutation

from GUA, the tyrosine tRNA anticodon) were deaminated to I, the resulting anticodon IUA ("topaz") would be able to read the codons UAA, UAC and UAU, but not UAG. UAC and UAU code for tyrosine. In order that such ochre-specific suppressors should not cause gross misreading, therefore, they are expected to insert tyrosine. This expectation has been borne out (Gilmore et al., 1968, 1971). If deamination occurs far more readily in yeast than in bacteria (inosine being far more prevalent in yeast than in E. coli tRNA) the lack of bacterial ochre-specific suppressors may be explained.

An alternative ochre-specific anticodon may be SUA ("sepia"), where S is a 2-thiouridine derivative present in yeast tRNA (Yoshida et al., 1970) which pairs only with A in the third pairing position. Mutation of a GUA anticodon to UUA, followed by enzymic modification could produce an SUA anticodon. If such modification of UUA always occurred, the lack of suppressors able to recognise both UAA and UAG would be explained (Gilmore et al., 1971).

The number of super-suppressor loci is large (Gilmore, 1967; Hawthorne and Mortimer, 1968). Due to the high redundancy of tRNA genes postulated for yeast (Schweizer et al., 1969), a great number of potential suppressor loci may exist. Since there are 8 species of tRNA whose anticodon can mutate by a single base change to the amber anticodon, an estimate of 6 cistrons per tRNA species (Schweizer et al., 1969) predicts 48 possible loci for amber suppressors alone. The actual number of loci available for mutation to suppressor genes is restricted by the dispensability and efficiency of each particular tRNA transcribed.

Due to the large number of suppressor loci and the variation in

phenotypic expression exhibited by many suppressors (discussed below), classification schemes based on suppression patterns alone have been found inadequate for the definitive identification of suppressor genes. Precise identification of a suppressor is possible only through a combined study of its suppression pattern and map position (Hawthorne and Mortimer, 1968). To date, 19 suppressor loci have been mapped (Hawthorne and Leupold, 1974).

Several different classification schemes have been presented (Inge-Vechtomov, 1965, 1966; Magni and Puglisi, 1966; Gilmore and Mortimer, 1966; Gilmore, 1967; Hawthorne and Mortimer, 1968; Hawthorne, 1969b) the most extensive being that shown in Hawthorne and Leupold (1974). Most classification schemes are expected to group suppressors into classes according to the codon(s) recognised, the amino-acid inserted and the efficiency of suppression. Such schemes are of limited value, as not only are they based upon an arbitrary, restricted set of nonsense alleles, but also one criterion of classification, that of suppressor efficiency, is subject to genic and cytoplasmic modification. In an ideal classification system, each suppressor within a class would be identical in all properties. Difficulties associated with scoring suppression efficiency mean that, in practice, different classes probably represent sets of iso-accepting tRNAs reading the same codon but with various degrees of efficiency; only gross differences in suppression are detectable and result in otherwise identical suppressors being placed in different classes. Some evidence for the identity of all the suppressors comprising a single class exists: all 7 super-suppressor genes of Class I set 1 and one of Class II of Gilmore's classification

scheme (Gilmore, 1967) cause insertion of the same amino-acid, tyrosine (Gilmore et al., 1968, 1971). The lower redundancy of tRNA genes in E. coli means that such classification problems are not encountered in this organism.

Yeast super-suppression is further distinguished from bacterial nonsense suppression by the nature of the modifiers of suppressor phenotype recovered. Suppression efficiency may be influenced by genic and cytoplasmic background factors which affect the balance between termination and translation at the nonsense codon. Nuclear and cytoplasmic mutants which modify suppression efficiency have been isolated. However, mutants analogous to the extensively investigated str A and ram mutants of bacteria (reviewed by Gorini, 1970), which restrict or enhance suppression efficiency via altered 30s ribosomal proteins, have not yet been identified in yeast.

The first modification system studied in depth in Saccharomyces cerevisiae was that of the cytoplasmically-inherited 'psi' factor (Cox, 1965). The psi system was originally postulated (Cox, 1965) to explain the occasional absence of the suppressed $ad_{2.1}$ phenotype in the presence of one of its suppressors, SUQ5. This apparent reversion to the red, adenine-requiring phenotype was stable through mitosis. Outcrossing of such unsuppressed haploids usually led to restoration of the suppressed phenotype in the diploid and normal segregation of the suppressed phenotype in the ascospore clones derived from it. The apparent 'de-suppressed' phenotype did not re-appear. However, crosses between independently arising de-suppressed haploids failed to restore the original white, suppressed phenotype, either in the diploids or in their haploid progeny. Furthermore,

additional crosses established that 2 other nonsense alleles normally suppressible by SUQ5, $his_{5.2}$ and $lys_{1.1}$, also exhibited a de-suppressed phenotype under conditions of $ad_{2.1}$ de-suppression.

In order to explain the absence of SUQ5 expression and the non-Mendelian pattern of inheritance of the de-suppressed phenotype, Cox (1965) postulated that expression of SUQ5 was dependent on certain self-replicating cytoplasmic particles, which he termed "psi". Expression of super-suppressor activity occurs in psi+ strains. Mutation of psi+ to psi- results in failure of SUQ5 to be expressed and thus appearance of the de-suppressed phenotype.

By analogy with the cytoplasmically-inherited ρ factor (B. Ephrussi and H. Hottinguer, 1951), which determines respiratory competence, the psi- cytoplasmic state is 'neutral' while the psi+ state is 'suppressive', (Ephrussi et al., 1955), thus accounting for the elimination of the psi- phenotype when such strains are outcrossed to psi+ strains.

From the above experiments, little could be deduced regarding the manner in which the psi system influences super-suppression. Further insight into both the mode of action and the mechanism of inheritance of psi followed from later studies.

The inheritance and maintenance of psi factors is controlled by a nuclear gene. This was concluded from experiments with certain UV-induced de-suppressed mutants of an $ad_{2.1}$ SUQ5 psi+ strain (Young and Cox, 1971). One such mutant displayed an unusual pattern of inheritance when outcrossed: crosses to $ad_{2.1}$ SUQ5 psi- haploids resulted in a red, unsuppressed diploid which, on sporulation, gave rise to 4 unsuppressed ascospore clones; crosses to $ad_{2.1}$ SUQ5 psi+

haploids resulted in unsuppressed, pink diploids which sporulated to give irregular segregation of mutant phenotypes, the non-suppressed phenotype occurring more frequently than the suppressed. Further crosses of the 4 red, non-suppressed spores of a tetrad of the latter diploid to a white, $ad_{2.1}$ SUQ5 psi+, followed by analysis of the haploid progeny, showed that 2 of the diploids gave rise to an excess of red, unsuppressed haploids, and 2 produced a majority of white, suppressed haploids. Such results could only be interpreted in terms of mutation of a nuclear gene in the original de-suppressed strain. This mutation, R, presumably prevents the expression of psi+ particles in psi+/psi- diploids, and must therefore be dominant to its wild-type allele. R also causes the slow, progressive elimination or mutation to inactivity, of psi+ particles during zygote division, as shown subsequently by the increase in percentage of red spores with time before meiosis of R/+ zygotes. The suppressed, white haploids derived from such a diploid must contain the wild-type allele of R and psi+ particles not eliminated by R before meiosis.

The method by which the nuclear gene exerts its control of the psi system is unknown. The most feasible explanation, accounting for the dominance of R, is that the wild-type allele of R codes for a repressor of psi function and replication. Mutant repressor produced by R would be unable to recognise the apo-repressor normally inactivating wild-type repressor. The psi system would thus be continually repressed.

No effect of R on the cytoplasmically-inherited rho or erythromycin resistance geneswas observed. Later experiments also demonstrated

independent inheritance of these mitochondrial genes and psi factors (Young and Cox, 1972).

Further information on the mode of action of the psi system comes from studies of its interaction with other super-suppressors. Evidence presented by Cox (1971) suggests that psi factors influence suppressor expression by their effect on suppression efficiency.

The ochre-specific super-suppressor SQ2 segregates as a recessive lethal in psi+ strains. Psi- diploids and haploids containing SQ2 are viable and suppressed. Expression of 4 Class I super-suppressors was tested in psi- and psi+ strains. All 4 were viable and expressed in psi- diploids and haploids. Zygotes produced by mating these suppressors to psi+ haploids were abnormal: some lysed during initial divisions while others grew only very slowly; all were unable to sporulate. No effect of psi status was detected with 2 Class III suppressors (Gilmore, 1967) or with the amber-specific allele of SQ2.

Consideration of the above interactions led to the hypothesis that the psi status of the cell influences the efficiency of suppression, thereby indirectly affecting the viability of some suppressors. Two assumptions were made: the psi+ state increases, and psi- decreases, suppression efficiency proportionately for each suppressor; too great an efficiency is lethal. The preceeding results can then be interpreted as follows: SQ5 is a weak suppressor; its efficiency is sufficiently high in psi+ strains to allow expression of ochre suppression, but too low for suppression to be detectable in psi- strains. SQ2 is more efficient than SQ5: it is expressed in psi- haploids and diploids and in psi+ diploids. In psi+ haploids, however, the efficiency of SQ2 is too great and therefore lethal.

SQ2 may be viable in psi+ diploids due to the relatively lower proportion of SQ2 product per protein synthesized compared with psi+ haploids. The 4 Class I suppressors tested are still more efficient. This is deduced from their suppression in psi- strains but lethality even in psi+ diploids.

The fact that the amber suppressing allele of SQ2 is not lethal in psi+ strains may indicate the absence of UAG as a normal stop codon.

It is unclear whether psi fails to affect the efficiency of Class III suppressors or whether its interaction is not measurable on the criteria used. The level at which the psi system exerts its effect is therefore unknown; suppression efficiency in general or suppression mediated only by specific suppressors may be affected.

The nature of psi factors is also unknown; any one of the many components of the protein-synthesizing system may be implicated.

Evidence supporting the above conclusion that psi affects suppression efficiency came from the work of McReady and Cox (1973) on nuclear modifiers of suppression efficiency. Mutations at 8 loci were shown to reduce SQ2 suppression efficiency in an $ad_{2.1}$ can_{1-100} $lys_{1.1}$ $try_{1.1}$ psi- strain. Reduction in suppression efficiency by such 'antisuppressor' genes was revealed as a lack of $ad_{2.1}$ and can_{1-100} suppression. Suppression of $lys_{1.1}$ and $his_{5.2}$ was subsequently found to be reduced to a lesser degree.

The lethality of SQ2 in psi+ haploids was abolished in the presence of an antisuppressor gene. Suppressor activity was detected in these strains.

The leaky suppression of $lys_{1.1}$ and $his_{5.2}$ alleles and the

viability of SQ2 psi+ in the presence of an antisuppressor, indicate that both the psi- state and antisuppressor mutations reduce super-suppressor efficiency.

Other nuclear mutations whose gene products modify suppressor expression have been isolated. Gorman and Gorman (1971) reported the isolation of a mutant, $sin_{1.1}$ (suppressor-interacting), which prevented suppression by 3 allele-specific suppressors of the missense mutant $his_{2.1}$. $Sin_{1.1}$ was recessive when tests for suppression were performed on media containing glucose as the carbon source, but dominant when glycerol replaced glucose. That the interaction of $sin_{1.1}$ gene products was specific for suppressor-tRNA was suggested by the improved growth rate of suppressed strains in the presence of $sin_{1.1}$ and its lack of effect in non-suppressed strains. $Sin_{1.1}$ thus exhibits some of the characteristics of str A mutants in bacteria (Gorini, 1969).

Mutations restricting S5 suppression of the ochre alleles $ad_{2.37}$, $ad_{2.105}$ (Soilda and Inge-Vechtomov, 1966) and $ad_{2.1}$ (Inge-Vechtomov, 1967) have been reported.

Hawthorne (quoted in Hawthorne and Leupold, 1974) has isolated a dominant antisuppressor, SIN 2, of certain susceptible amber suppressors. Also reported (Hawthorne, 1967; Hawthorne and Leupold, 1974) are a class of modifiers which not only restore activity to the amber suppressor SUP5-a' restricted by SIN 2, but also increase the efficiency and action spectrum of ochre-specific suppressors unaffected by SIN 2. A Class I ochre-specific suppressor in conjunction with such an 'allosuppressor' can suppress amber alleles. By themselves, allosuppressors are weak, recessive suppressors with a very limited action spectrum; they may be alleles of omnipotent

suppressors. As mentioned previously, omnipotent suppressor genes may code for non-tRNA components of the translation machinery such as ribosomal proteins.

Modification of suppressor phenotype by factors displaying Mendelian or non-Mendelian inheritance have been described. No modifier has been studied sufficiently to determine how it functions. Further investigations with such systems is likely to yield valuable information on various aspects of protein synthesis.

It was originally intended to study the effect of post-UV liquid-holding treatment on mutation at missense, nonsense and super-suppressor loci. It was thereby hoped to gain insight into the unexplained observation that the majority of spontaneous revertants in a strain multiply-marked with nonsense mutants are due to super-suppression, while UV-induced revertants are almost all due to true reversion or single allele-specific suppressors. A diploid homozygous for 3 ochre alleles, $ad_{2.1}$, $arg_{4.17}$ and $lys_{1.1}$ was constructed. Initial results were unexpected and required explanation before the above project could be continued. The problems encountered will be detailed in "Results". In brief, it was found that suppressed $ad_{2.1}$ strains exhibited, on adenineless medium, a "leaky" growth phenotype. The degree of "leakiness" present was subsequently found to be amenable to manipulation by the use of various simple growth techniques. It was considered that, if the penetrance of the suppressor were, by this means, being modified, this system could prove useful in the study of various aspects of suppression and protein synthesis in a eucaryotic organism. Indeed, even if the phenomena observed were not manifestations of alterations occuring in the suppression system, they were thought

interesting to investigate from the standpoint of genetic - environmental interactions. Investigations of the original unexpected phenomena thus produced results of sufficient interest to justify their becoming the basis of the present thesis.

CHAPTER II

MATERIALS and METHODS

(i) Strains

69/1: a diploid of genotype:

$$\frac{a}{\alpha}\ \frac{ad_{2.1}}{+}\ \frac{arg_{4.17}}{+}\ \frac{lys_{1.1}}{+}\ \frac{his_{5.2}}{+}\ \frac{try_{5.48}}{+}\ \frac{leu_{1.12}}{+}\ \frac{met_{1.1}}{+}\ \frac{+}{rad_{1.1}}$$

This diploid had been derived from the multiple-auxotroph X1687-16c, obtained from Dr R.K. Mortimer, and the prototrophic, UV-sensitive strain $rad_{1.1}$. The $ad_{2.1}$, $arg_{4.17}$, $lys_{1.1}$, $his_{5.2}$ and $try_{5.48}$ alleles are ochre nonsense mutants, all super-suppressible by Class I supersuppressors.

69/1/3: a haploid of genotype:

$ad_{2.1}\ arg_{4.17}\ lys_{1.1}\ his^{+}\ try_{5.48}\ leu_{1.12}\ met_{1.1}\ rad^{+}$

derived from 69/1.

69/1/9: a haploid of genotype:

$ad_{2.1}\ arg_{4.17}\ lys_{1.1}\ his_{5.2}\ try^{+}\ leu_{1.12}\ met_{1.1}\ rad^{+}$.

4c: a diploid obtained by crossing 69/1/9 with 69/1/3.

2885-32B: a haploid of genotype:

$\underline{a}\ ad_{5,7-101}$, $lys_{1.1}\ leu_{2.1}\ ura_{4.1}\ met_{8.1}\ try_{1.1}$,

kindly supplied by Dr D.C. Hawthorne. The $ad_{5,7-101}$, $lys_{1.1}$, $leu_{2.1}$ and $ura_{4.1}$ alleles are ochre nonsense, while the $met_{8.1}$ and $try_{1.1}$ alleles are amber nonsense mutants.

Other strains in use in this laboratory were occasionally used and will be described where appropriate in the text.

(ii) Culture Media

Yeast Extract Peptone Dextrose (YEPD)

YEPD slants were used for routine maintenance of stocks stored at 4°C. This medium comprised bacto-peptone, glucose and agar each at 2.0%, yeast extract powder at 0.5% and 20mg/l adenine sulphate.

Glucose Nutrient Broth (GNB)

GNB, inoculated with yeast and shaken at 32°C, was used routinely for the production of exponential or stationary phase cultures. The medium consisted of 0.35% bacto-peptone, 0.4% yeast extract powder, 1% glucose, 0.1% ammonium sulphate, 0.2% potassium dehydrogen ortho-phosphate, 0.1% magnesium sulphate and 20mg/l adenine sulphate.

Yeast Extract Agar (YEA)

YEA was used both as the standard solid complete medium and also as a presporulation medium. It comprised 2.3% yeast extract agar, 1.5% glucose and 20mg/l adenine sulphate.

Glucose Nutrient Agar (GNA)

GNA was used initially as a presporulation medium. It comprised 5% glucose, 1.3% nutrient broth, 1% yeast extract powder and 1.5% agar.

Potassium Acetate (PA)

PA was used to induce sporulation. It consisted of 1% potassium acetate, 0.25% yeast extract powder and 3% agar.

Solid and Liquid Minimal Medium (MIN)

Solid MIN was occasionally used, without added supplements, as a crossing medium. With supplements added, it was routinely used as omission medium for selecting revertants and testing for auxotrophic requirements. It consisted of 4% glucose and 0.67% Difio Yeast Nitrogen Base without amino-acids, solidified with 2% agar.

Liquid MIN, with particular supplements added, was used as liquid omission medium.

Synthetic complete medium was not used. Instead, MIN supplemented as required for the unsuppressed strain under consideration, referred to as "+All", was utilised.

For 69/1 - derived strains, +All supplementation was as follows:

adenine sulphate	20mg/l
L-arginine monohydrochloride	20mg/l
L-lysine monohydrochloride	20mg/l
L-histidine monohydrochloride	10mg/l
L-tryptophan	20mg/l
DL-leucine	60mg/l
methionine	20mg/l

For 2885-32B, uracil at 20mg/l was also added to the above supplements.

For strains auxotrophic for serine, DL serine at 37.5mg/l was added to the above supplements.

Omission media for each strain was the respective +All lacking a single supplement. This supplement determined the nomenclature of the omission medium e.g. -AD (- adenine) for 69/1 - derived strains is minimal supplemented with

arginine, lysine, histidine, tryptophan, leucine and methionine only, but -AD for 2885 strains contains uracil in addition to these.

Any other relevant supplemented-minimal media used will be described where necessary in the text.

Potassium Dihydrogen Orthophosphate (KH_2PO_4) and Sodium Citrate Buffers

A 0.067M solution of KH_2PO_4 at pH 5.2 was used regularly as a suspending medium.

Since growth in liquid minimal often led to "clumping" of cells, citrate buffer was used as the suspending medium in such experiments because of its "declumping" properties. Citrate buffer (pH 5) consisted to 0.9% trisodium citrate and 0.3% citric acid.

(iii) Phenotype Determination

1) Spot-testing

Spot-tests were used initially for tetrad analysis. The method was as follows: ascospore colonies were suspended in 0.5ml 0.067M KH_2PO_4 and spotted onto appropriate omission media using a $\frac{1}{4}$" diameter metal rod. This technique was found to be time-consuming and unsatisfactory for determining the proportion of cells of a population giving rise to growth. Streak-testing was therefore used for tetrad analysis in all but the first few experiments.

2) Streak-testing

Streak-testing was used for tetrad analysis and routine testing of revertants. 1cm streaks were applied to appropriate media, care being taken to streak approximately equal quantities on each plate.

3) Replica-Plating

The Lederberg technique of replica-plating (Cavalli-Sforza, Lederberg, 1953) was used for screening large numbers of colonies for particular phenotypes, e.g. for random spore analysis and revertants induced by UV. Ambiguous results were checked by test-streaking.

Replica-plating was found an unsatisfactory technique for determining the presence or absence of suppression, especially for the $ad_{2.1}$ allele (see Results) or where mutants are "leaky". In these cases, test-streaking was preferred as the only method allowing distinction between growth of the whole population and growth originating from a small number of cells.

(iv) Genetic Analysis

1) Diploid Formation

Wherever possible, diploids were produced by cross-streaking haploids of opposite mating-type and of differing auxotrophic requirements on omission medium permitting diploid growth only. Diploid colonies arise from the cross-streaked region after 4-7 days' incubation at 32°C.

In cases where no such auxotrophic requirements were present, diploids were produced by cross-streaking approximately equal quantities of a and α strains on YEA. Diploid formation in the area of cell mixing was assessed after 1-2 days by streaking a sample on to PA. After incubation (see below), a suspension of cells in water

examined for asci at 200x magnification, revealed the % sporulation of the sample. The extent of diploid formation in the sample and the nature of the experiment determined whether or not single diploid colonies were subsequently isolated by restreaking from the cross-streak prior to sporulation.

2) Sporulation

Diploids were streaked onto presporulation medium and incubated at 32°C for 2-3 days. (Incubation on YEA for 3 days was found to be as effective as incubation on GNA for 2 days in inducing subsequent sporulation, and was used in all but the first few sporulation experiments). A loopful of the diploid was then spread thinly over the surface of a PA plate. Incubation at 25°C for 3-4 days resulted in the formation of mature asci.

3) Ascus Digestion

Random ascospores or discrete meiotic tetrads were recovered after complete or partial digestion, respectfully, of the walls of mature asci. This was achieved as follows:- 0.02 ml snail digestive juice (Koch-Light) was added to a loopful of sporulating culture suspended in 1.0 ml 0.067M KH_2PO_4, and the mixture incubated at 32°C for either 30 or 90 minutes. Before further manipulation, the degree of digestion was first checked by microscopic examination of a sample of the digesting suspension streaked on YEA.

For random spore analysis, the suspension was plated at appropriate dilutions on YEA or selective medium, whichever appropriate.

For ascus dissection, a loopful of suspension was streaked along one side of a thick, oven-dried plate of YEA. Micro-manipulation was accomplished using a Singer micro-manipulator fitted with a dissecting loop of 15 micron diameter made from Pyrex glass tubing in a de Fonbrune Microforge. A Bausch and Lomb Dynazoom Laboratory Research Microscope fitted with a 10x objective, 10x eyepieces (wide field) and 2x setting of the 'zoom' lens completed the dissection apparatus.

Suitably-digested tetrads were selected and the 4 spores placed at 4mm intervals in a line at right angles to the streak. Separate sets of tetrads were placed 5mm apart. Plates were incubated at 32°C unless otherwise stated.

4) Tetrad and Random-Spore Analysis

a) Tetrad Analysis

Tetrads yielding 4 viable ascospores were tested for their auxotrophic requirements. Occasionally, when ascospore viability was very low, triplets or doublets of viable spores had to be used. Ascospore clones were tested directly from dissection plates (see above).

b) Random Spore Analysis

Where haploids could not be distinguished on the basis of colour (as when ad_1 or ad_2 mutants were segregating), small colonies were selected from random spore plates and picked with sterile cocktail sticks onto YEA plates. After 1-2 days incubation at 32°C, these YEA master plates were replica-plated onto appropriate omission media using the standard Lederberg velvet pad technique (see above).

CHAPTER III

RESULTS

The rationale behind the use of diploid 4c in the investigation of UV-induction of super-suppressors was based on the knowledge of of the higher UV-resistance of diploids compared with haploids, allowing relatively high UV doses to be applied while retaining high survival levels, and on the premise that the typical dominance of super-suppressors would allow their recovery in heterozygous form.

Preliminary experiments to recover spontaneous reversions of the auxotrophic requirements of 4c were carried out. The majority of spontaneous revertants were expected to be due to Class 1 super-suppressors, which can suppress all the ochre alleles of 4c, as is the case in the haploid strain from which 69/1 was derived (Queiroz, 1971). However, no such super-suppressors appeared to arise in 4c, as in no case was the $ad_{2.1}$ allele suppressed. After prolonged incubation of -AD replica-plates, very small white papillae appeared on the majority of those red imprints believed to contain a super-suppressor by their simultaneous independence of arginine and lysine. This phenomenon was investigated, and led to the results reported here. However, the essential feature of the system studied, its variable expression, was not appreciated at the beginning of the investigation. Only after several sets of experimental observations, internally consistent at any one time, were found to differ when repeated at different times, did this feature become clear. Section I of the Results presents the experimental evidence

Section I: Initial Observations

a) Cell Morphology of 69/1-Derived Strains

Cells of 69/1-derived strains, when microscopically examined, were observed to be of irregular size. Unsuppressed 69/1/3 and 69/1/9 cells varied in size from that of a normal haploid to approximately 10 times this size. Unsuppressed 4c cells varied from one to 5 times the size of normal diploid cells.

The presence of supersuppressors appeared to exaggerate this abnormality in that a higher frequency of large cells were present in suppressed haploids and that suppressed 4c strains exhibited a higher frequency of cells up to about 10 times the size of those of a normal diploid.

This phenotype varied according to the isolate, suppressor and medium used. Such enlargement typifies cells whose growth and division are poorly synchronised. Gilmore (1967) reported a similar phenotype for haploid cells bearing 2 supersuppressors. Plates 1a,b illustrate the extreme form of the phenotype, a colony of a suppressed 4c isolate, compared with that of a normal diploid, 69/1; both photomicrographs were taken after overnight growth of single cells on -AD plates.

b) Recovery of Super-Suppressors

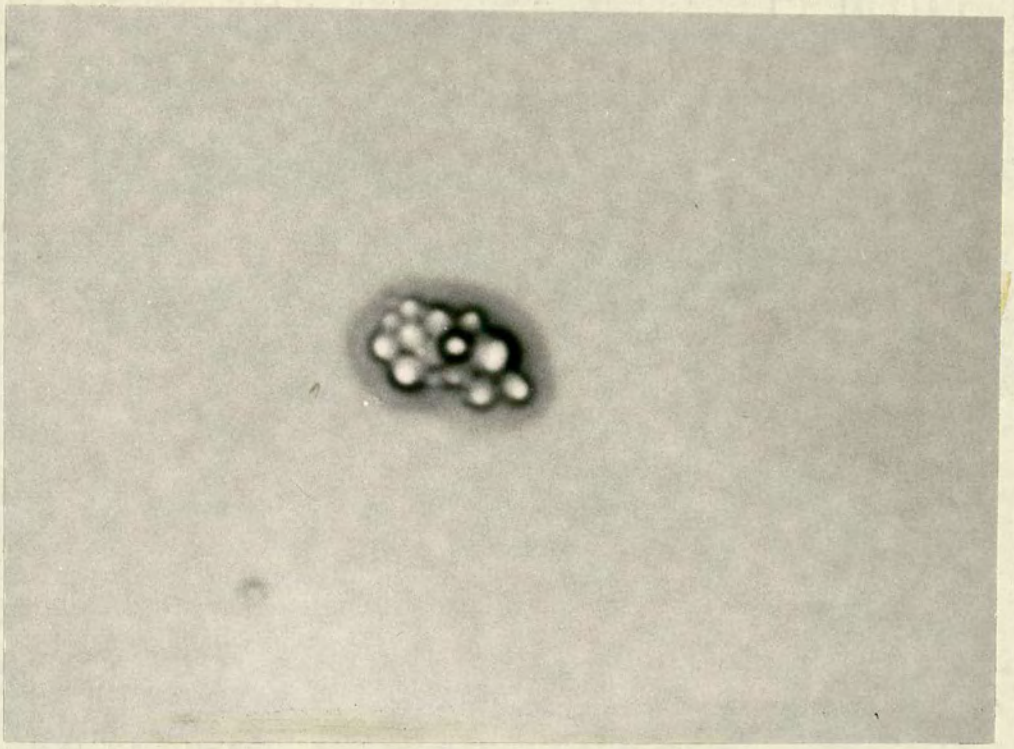
i) Diploid 4c

Experiments were carried out to determine the proportion of revertants due to suppressor mutations in untreated and UV-irradiated cultures of 4c. Washed suspensions of cells were treated and 0.1ml aliquots plated on omission media to recover revertants and on YEA

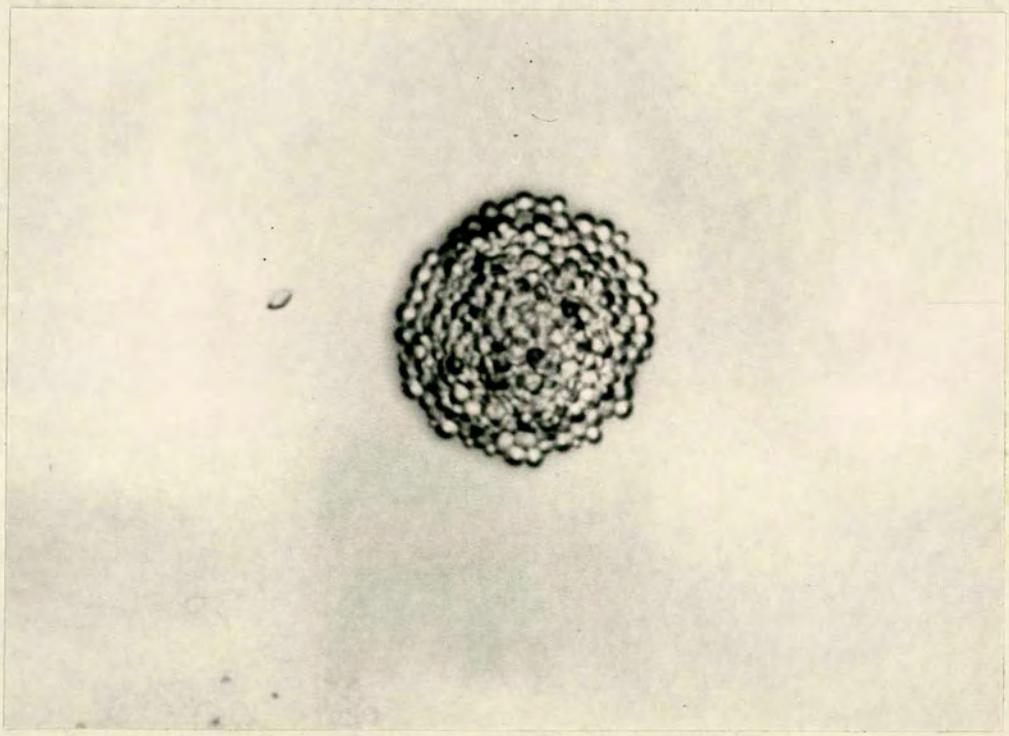
Plate 1 Photomicrographs of Diploid Colonies on -AD Medium

Both colonies arose from overnight growth of a single cell.

Plate 1



a. Aberrant morphology of a Suppressed 4c isolate
x 1750



b. Wild-type morphology of strain 69/1
x 1750

to assess survival. All revertants, or a large random sample where reversion frequency was high, were tested for growth on -AD, -ARG, -LYS and +ALL. Simultaneous reversion at 2 or more loci was considered to be evidence for the presence of a super-suppressor. Typical results are shown in Table 1.

The results show that most spontaneous revertants carried super-suppressors, while the majority of UV-induced reverants were non-suppressors. These findings agree with those of Queiroz (1971). No super-suppressors were recovered on -AD. When tested on -AD, the majority of suppressor-bearing strains gave the anomalous phenotype described above. Only true ad^+ revertants grew well on -AD.

It is proposed to identify super-suppressors isolated in the diploid by the symbol su_D.

ii) Haploids 69/1/3 and 69/1/9

That the ad^- allele in 4c was indeed suppressible was shown by testing spontaneous revertants of 69/1/3 and 69/1/9. Results of a UV-induction experiment using 69/1/3 are shown in Table 2.

Super-suppressors were recovered on all omission media, but their frequency on -AD was always very low. Most arg^+ lys^+ revertants were found also to grow to a variable extent on -AD. The ad^- allele was concluded to be suppressible and therefore almost certainly $ad_{2.1}$. Treatment with UV light induced true reversions but not super-suppressors. Spontaneous revertants of several other multiply-marked haploid derivatives of 69/1 were tested. All showed similar variable suppression on -AD.

Table 1

Treatment	% Survival	No. Revertants			No. Super-suppressors		
		-ARG	-AD	-LYS	-ARG	-AD	-LYS
Control	100	-	-	4	-	-	4
840 ergs/mm^2	5.24	206	38	95	-	-	-

Table 2

Treatment	% Survival	No. Revertants			No. Revertants Tested			No. Super-suppressors		
		-AD	-ARG	-LYS	-AD	-ARG	-LYS	-AD	-ARG	-LYS
Control	100	-	4	4	-	4	4	-	4	4
140 ergs/mm^2	84.3	94	269	208	62	62	62	-	2	2
420 ergs/mm^2	41.7	208	244	305	62	62	62	-	-	-

Super-suppressors isolated in a haploid will be identified by the symbol su_H.

c) Growth Phenotypes of Super-Suppressors

i) su_D Isolates

Super-suppressed 4c isolates grew well when replica-plated or streaked on -ARG and -LYS. In preliminary experiments, su_D-containing strains were found to be red in colour and not to grow on -AD plates within approximately 10 days of testing. Subsequently, however, a number of small white colonies arose from the background of red, non-growing cells. No such colonies appeared from the background of unsuppressed isolates. Only one or two colonies usually arose from a single imprint or test-streak. Minute white papillae were seen on the surface of red suppressed streaks growing on -ARG and -LYS.

In later experiments, after storage of 4c in the refrigerator for some months, this behaviour was slightly altered in that small white colonies now appeared on some streaks after about five days incubation.

Data from many experiments indicated that the frequency and time of appearance of white colonies on -AD streaks varied, not only between different suppressor isolates, but also between separate tests of the same suppressor isolate.

Additional multiply-marked haploid derivatives of 69/1 were inter-crossed and a further 3 diploids formed. Suppressed isolates of all 3 behaved in the same manner as 4c.

ii) su_H Isolates

su_H strains grew well on -ARG, -LYS, -HIS and -TRY. Growth on -AD was extremely variable.

When first tested, both 69/1/3 and 69/1/9 grew somewhat poorly on -AD: growth was slow and often "leaky" in appearance (having discrete colonies growing over the background streak of non-dividing cells) but always sufficient to be classified, after 3-4 days, as positive suppression.

Later experiments suggested that 69/1/3 could no longer show definite $ad_{2.1}$ suppression. Subsequent tests revealed that this apparent change in behaviour pattern of 69/1/3 was merely a manifestation of the extremely variable $ad_{2.1}$ suppression phenotype found between different haploid isolates, between different suppressors tested at the same time, and between the same isolates and suppressors tested at different times.

iii) Other Isolates

su_H haploids were crossed to an unsuppressed haploid of opposite mating-type. The resulting $\frac{su_H}{+}$ diploids were tested for $ad_{2.1}$ suppression. In general, suppressed growth was worse than for haploid su_H strains, but better than for su_D-bearing diploids. Again, the extent of growth obtained on -AD was variable.

Several $\frac{su_H}{+}\ \frac{+}{su_D}$ diploids were constructed. When tested on -AD plates, growth ability, although variable, was generally greater than for $\frac{su_H}{+}$ strains.

As indicated above, growth of streaks on -AD plates was often

difficult to score, varying as it did from but one colony to complete confluence. In general, however, 3 main types of growth pattern were observed after approximately 5 days incubation:

a. Growth of one large colony at one or each end of the streak

b. Intermittant growth of small colonies along the streak

c. Confluent growth

These growth types are not rigidly defined; the growth phenotype depended considerably on the time between streaking and scoring. Thus streaks with colonies only at either end may also show a little intermittant growth if left for one or two additional weeks after the original scoring. Except where otherwise stated, -AD growth phenotypes described are those after approximately 5 days incubation.

d) Experiments to Eliminate Mechanisms other than Suppression as the Cause of Growth on -AD

An intermittant growth phenotype on -AD, particularly that described for su_D diploids, may be ascribed to any one of a number of possible causes. Among these are:

1) Suppression at low efficiency

2) Death of a proportion of su^+ cells, leading to release of nutrients into the surrounding medium allowing growth of the remaining live cells.

3) Some sort of "metabolic co-operation" effect where contact between suppressor-bearing cells enables growth which could not otherwise occur.

4) A high rate of true $ad_{2.1}$ reversion in the presence of a suppressor, or mitotic conversion of the suppressor to form $\frac{su_D}{su_D}$ diploids.

Possibilities 2) - 4) were rejected on the basis of the following experiments:

i) Reconstruction Experiment (Possibility 2)

An su_H-bearing haploid and an su_D-bearing diploid, both with a poor growth phenotype on -AD, were used. Each was streaked on the following media:

-AD

+ALL

-AD + lawn of UV-killed cells of same strain

+ALL + lawn of UV-killed cells of same strain

On subsequent incubation, both strains showed poor growth on -AD with or without a background of dead cells. It was concluded that live cells could not benefit from the presence of surrounding dead cells to an extent which resulted in visibly improved growth on -AD. Possibility 2) above was therefore rejected.

ii) Plating Experiment (Possibility 3)

Several suppressor-bearing haploid and diploid strains, and an ad^+ arg^+ haploid derivative of 69/1, were plated on -AD, -ARG and +ALL at a dilution giving about 200 colonies per +ALL plate. After 3 days, approximately equal numbers of colonies appeared on -ARG and +ALL for all strains and also on -AD for the ad^+ arg^+ haploid. For the other strains, wide variation was found in the time of appearance, size and number of colonies on -AD compared with +ALL. Most colonies had appeared by 5-6 days. Colony counts on -AD were 10-100% of those obtained on +ALL. These viability levels rule out any mechanism requiring cell contact as the cause of poor growth

of streaks on -AD, as the majority of colonies must arise from single, isolated cells. Possibility 3) above was therefore dismissed.

Possibility 4) also appeared extremely unlikely since, if all colonies on -AD arise via back mutation of $ad_{2.1}$, the mutator activity of the super-suppressors would have to be several orders of magnitude greater than that for any known mutator gene. Furthermore, restreaking such colonies on -AD did not immediately give rise to confluent growth (as described in Section II) as would be predicted for an ad^+ strain.

iii) Ascus Dissections

Ascus dissection experiments finally confirmed that growth on -AD was due to suppression, although, as will be seen, the preliminary results were misleading.

Red, suppressor-bearing isolates of 4c, and single white colonies derived from them on -AD plates, were used for initial ascus dissections. If both types of isolate carry a suppressor of variable expression, evidence of it should be obtained from both crosses. Unsuppressed 4c was also dissected. In the latter, segregations of auxotrophic markers were as expected (for examples, see Table 3). Three red, suppressed 4c strains, however, gave aberrant segregation patterns for all alleles in each of the 20 tetrads tested, with no clear segregation of a super-suppressor gene. An excess of prototrophs was usually obtained for all but the ad_2 locus, which invariably showed $0^+:4^-$ segregation. Examples of segregation patterns obtained are shown in Table 4.

A white colony from the -AD plate of each of the 3 suppressed

Table 3 Segregation in 5 Tetrads of Unsuppressed 4c

\- : no growth

\+ : growth

Table 3

Tetrad	Ascospore clone	-AD	-ARG	-LYS	-HIS	-TRY	+ALL
1	a	-	-	-	-	-	+
	b	-	-	-	-	-	+
	c	-	-	-	+	+	+
	d	-	-	-	+	+	+
2	a	-	-	-	-	-	+
	b	-	-	-	-	+	+
	c	-	-	-	+	+	+
	d	-	-	-	+	-	+
3	a	-	-	-	-	+	+
	b	-	-	-	+	+	+
	c	-	-	-	+	-	+
	d	-	-	-	-	-	+
4	a	-	-	-	+	-	+
	b	-	-	-	+	-	+
	c	-	-	-	-	+	+
	d	-	-	-	-	+	+
5	a	-	-	-	-	+	+
	b	-	-	-	+	+	+
	c	-	-	-	+	-	+
	d	-	-	-	-	-	+

Table 4 Aberrant Segregation in 5 Red Suppressed 4c Tetrads

- : no growth

+ : growth

Table 4

Tetrad	Ascospore clone	-AD	-ARG	-LYS	-HIS	-TRY	+ALL
1	a	-	+	+	+	+	+
	b	-	-	-	+	+	+
	c	-	+	+	+	+	+
	d	-	-	+	+	+	+
2	a	-	-	+	+	+	+
	b	-	-	+	+	+	+
	c	-	+	+	+	+	+
	d	-	+	+	+	+	+
3	a	-	-	+	+	+	+
	b	-	+	+	+	+	+
	c	-	+	+	+	+	+
	d	-	-	+	+	+	+
4	a	-	+	+	+	+	+
	b	-	-	-	+	+	+
	c	-	-	+	+	+	+
	d	-	+	+	+	+	+
5	a	-	+	+	+	+	+
	b	-	+	+	+	+	+
	c	-	-	+	+	+	+
	d	-	-	+	+	+	+

strains gave $2^+:2^-$ segregation of $ad_{2.1}$ in the total 23 tetrads tested (for examples, see Table 5), but, because of aberrant segregation of all other alleles, no conclusions could be drawn concerning the original suppressor genotype. The cause of the aberrant segregation is unknown. 4 $\frac{su_H}{+}$ diploids were constructed and dissected at approximately the same time as the above $\frac{su_D}{+}$ strains. In general, $\frac{su_H}{+}$ diploids grew to a slightly greater extent on -AD than did $\frac{su_D}{+}$ diploids. For all 17 tetrads tested, segregation of all alleles was that expected in the presence of a heterozygous suppressor. Table 6 shows examples of segregation patterns obtained.

It appeared, therefore, that aberrant segregation of $\frac{su_D}{+}$ diploids was in some way correlated to the fact that the suppressor was isolated in the diploid state and an attempt was made to investigate this aspect further. Unfortunately, after prolonged storage in the refrigerator, the $ad_{2.1}$ suppression properties of 4c altered, as noted above (see c) i)). At the same time, segregation of auxotrophic markers in suppressed strains became explicable in terms of a single segregating super-suppressor gene. Many attempts were made to recover suppressed diploids displaying the original aberrant segregation phenotype, all without success. In all, 6 suppressed 4c isolates and 5 other 69/1-derived multiply-marked suppressed diploids were dissected. A total of 125 tetrads were test-streaked. Growth capacity of different ascospore clones on -AD varied, but was generally greater than in the parent diploid. This may indicate a dosage effect for suppressor gene product. Clear segregation of a suppressor gene demonstrated that the abnormal

Table 5 Aberrant Segregation in 3 Tetrads of a White Suppressed 4c Colony on -AD

- : no growth

+ : growth

Table 6 Segregation in 5 Tetrads of a $su_{H/+}$ Diploid

- : no growth

+ : growth

Table 6

Tetrad	Ascospore clone	-AD	-ARG	-LYS	-HIS	-TRY	+ALL
1	a	+	+	+	+	+	+
	b	-	-	-	+	+	+
	c	-	-	-	-	+	+
	d	+	+	+	+	+	+
2	a	-	-	-	-	-	+
	b	+	+	+	+	+	+
	c	-	-	-	+	+	+
	d	+	+	+	+	+	+
3	a	-	-	-	+	+	+
	b	-	-	-	-	-	+
	c	+	+	+	+	+	+
	d	+	+	+	+	+	+
4	a	+	+	+	+	+	+
	b	-	-	-	+	-	+
	c	-	-	-	-	-	+
	d	+	+	+	+	+	+
5	a	-	-	-	-	-	+
	b	+	+	+	+	+	+
	c	-	-	-	+	-	+
	d	+	+	+	+	+	+

growth phenotype originally observed for suppressed 4c strains on -AD (and still apparent to a slightly reduced extent) was indeed probably due to weak super-suppression rather than suppressor-induced true reversion of $ad_{2.1}$ or mitotic conversion of su_D.

e) Failure to Detect Antisuppressor Factor

The possibility that the low level of $ad_{2.1}$ suppression observed could be due to genic (McCready and Cox, 1973) or cytoplasmic (Cox, 1965) antisuppressor factors was considered. To test this, 69/1/3 and 69/1/9 were outcrossed to several unrelated auxotrophic strains.

A chromosomal antisuppressor gene would be recovered in at least 50% of multiply-marked haploid progeny, while a recessive cytoplasmic antisuppressor may be eliminated altogether.

The following crosses were made:-

69/1/3 x ser^-

69/1/3 x $ad_{2.1}$ leu^-

69/1/9 x thr^- $ad_{2.1}$

69/1/9 x $ad_{2.1}$ leu^-

It will be noticed that both 69/1/3 and 69/1/9, which cross together, also appear to cross with the same haploid strain, $ad_{2.1}$ leu^-. This abnormality in mating-type control was noted several times during the course of this study.

Several multiply-marked progeny of the above crosses were isolated and spontaneous revertants tested on appropriate omission media.

All crosses except 69/1/3 x ser^- gave rise to progeny showing the same $ad_{2.1}$ suppression phenotype as the 69/1-derived parent.

The cross 69/1/3 x ser^- produced interesting results. The majority of spontaneous revertants in 3 different haploids were due to super-suppressors, as expected. $Ad_{2.1}$ suppression was poor. 5 other haploids, however, exhibited a low reversion frequency at all loci, all revertants recovered being single-site. Subsequent investigation with these haploids showed that suppressors do arise after prolonged incubation of omission plates. It is clear that the factors responsible for this abnormal behaviour must have been introduced from the ser^- parent, as it was not exhibited by any other than the above 5 haploids.

There is thus no evidence for either a chromosomal antisuppressor gene or recessive cytoplasmic antisuppressor factors.

A dominant mutant reducing suppressor efficiency may have arisen in 4c itself, accounting for reduced $ad_{2.1}$ suppression in the diploid compared with the component haploids 69/1/9 and 69/1/3. This seems unlikely as it would have to be postulated that the same event also occurred in 3 different 69/1-derived diploids and several zygotes of the cross 69/1/9 x 69/1/3, all of which, when suppressed, show a similar phenotype on -AD.

However, to eliminate this possibility altogether, 4c and another 69/1-derived diploid were dissected. 2 tetrads of each were used. Spontaneous revertants of each ascospore clone were selected and test-streaked. Once again, growth ability on -AD varied between the various haploid isolates, but was in all cases poor. No $2^+:2^-$ segregation of ability to express $ad_{2.1}$ suppression was apparent, so the possibility of a chromosomal antisuppressor in 4c was rejected.

Two strains unrelated to 69/1, ad_1 $leu_{1.12}$ $arg_{4.17}$ and thr^- $ad_{2.1}$, were used to construct the diploid $\frac{ad_{2.1}}{ad_{2.1}} \frac{arg_{4.17}}{arg_{4.17}}$. Spontaneous arg^+ colonies were test-streaked on -AD and -ARG. The majority grew very poorly on -AD. No ad^+ revertants were recovered. The conclusion was drawn that very poor $ad_{2.1}$ suppression, subject to wide variation in expression, may be characteristic of most $ad_{2.1}$ homozygous diploids.

f) Summary

$Ad_{2.1}$ suppression is weak in haploids and very weak in $ad_{2.1}$ homozygous diploids. Ability to grow on -AD varies widely between suppressors. It appeared that expression of any particular suppressor is influenced by unknown background factors which may change with time. The conclusion is drawn that $ad_{2.1}$ suppression, as revealed by growth on -AD, is not always an inevitable phenotypic consequence of the presence of a genetic suppressor known to be capable of $ad_{2.1}$ suppression, but depends also on a number of genetic or environmental factors. An attempt to investigate the nature and mode of action of these proposed modifying factors follows.

Section II: Experiments to Control Expression of Suppressors

a) Preliminary Observations

It was noticed that, on restreaking the -AD growth of suppressed haploids onto -AD, its growth phenotype changed slightly. The change was, in most cases, towards increased growth ability. For instance, growth of a suppressed haploid, present only at either end of a -AD streak, when restreaked onto -AD, produced once again growth mainly at either end of the streak, but also, after further incubation, growth throughout the whole streak in the form of minute, isolated colonies.

The observations that:-

1) Restreaking from -AD alters subsequent growth capacity on -AD

and 2) Plating on -AD results in a variable, slow growth rate with viability also variable but usually less than on +ALL plates (see Section I, d) ii) above) were made with very many suppressed haploid and diploid strains.

A preliminary experiment designed to detect any correlation between these 2 observations was carried out. 3 different suppressed strains originally derived from 69/1, 'a', 'b' and 'c', of differing growth capacity on -AD, were used. Suspensions of cells in 0.06M KH_2PO_4 from -AD and +ALL plates were made. Appropriate dilutions of these suspensions were plated on -AD, -ARG and +ALL, to give approximately 200 colonies per +ALL plate. The number of days before colonies were first distinguished was noted. The results are shown in Table 7.

Table 7 Correlation Between Growth Phenotype on -AD, Growth Medium Used and Growth Rate on Subsequent Plating

++++ : confluent growth

+++ : growth at ends and intermittantly in middle of streak

++ : growth only at end(s) of streak

Table 7

Strain	Growth Phenotype on -AD	Medium from which cell suspension made	No. of Days Between Plating and first appearance of colonies		
			-AD	-ARG	+ALL
a	+++++	-AD	2	2	2
		+ALL	2	2	2
b	+++	-AD	2	2	2
		+ALL	5	2	2
c	++	-AD	7	2	2
		+ALL	-	3	2

Despite the limited nature of the experiment, several tentative conclusions were possible and used as the basis for further experiments. These conclusions were:

1. The growth rate on -ARG (as measured by time between plating and first appearance of colonies) is not significantly different from that on +ALL for strains 'a' and 'b'. Strain 'c' may grow more slowly on -ARG than on +ALL.

2. The better the original growth of -AD streaks, the faster the growth on subsequent plating on -AD. (Strain 'a' grows on -AD after 2 days whereas strain 'c' takes 7 days).

3. A suspension of cells from -AD grows more quickly when subsequently plated on -AD than does a suspension of cells from +ALL. (For strain 'b', 2 days are taken and for strain 'c' 7 days are taken by cells from a -AD suspension; respective times for cells from +ALL are 5 days and infinity).

Conclusions 2 and 3 led to the following tentative hypothesis: the ability of a cell to grow by super-suppression on -AD depends on certain background (possibly cytoplasmic) conditions. Conditions favourable for super-suppressor activity to an extent which allows growth on -AD are present in a certain proportion of cells of a population. This proportion determines the growth phenotype on -AD. Selection of cells growing on -AD increases the proportion of cells with the ability to exhibit $ad_{2.1}$ suppression. Continuation of such selection may eventually lead to an entire population of cells able to grow well on -AD. Such a selected strain would appear as a normal ad^{+} strain.

If such selection were possible, it may occur via increased suppression efficiency. This increase in efficiency may only be detected by its effect on the $ad_{2.1}$ allele, since, as has been suggested by previous work (Cox, 1965; McReady and Cox, 1973), the $ad_{2.1}$ allele is "less easily suppressed" than either $lys_{1.1}$ or $arg_{4.17}$. As a result, growth on -LYS and -ARG is normal even at a low suppression efficiency, any increase of which will therefore have no observable effect.

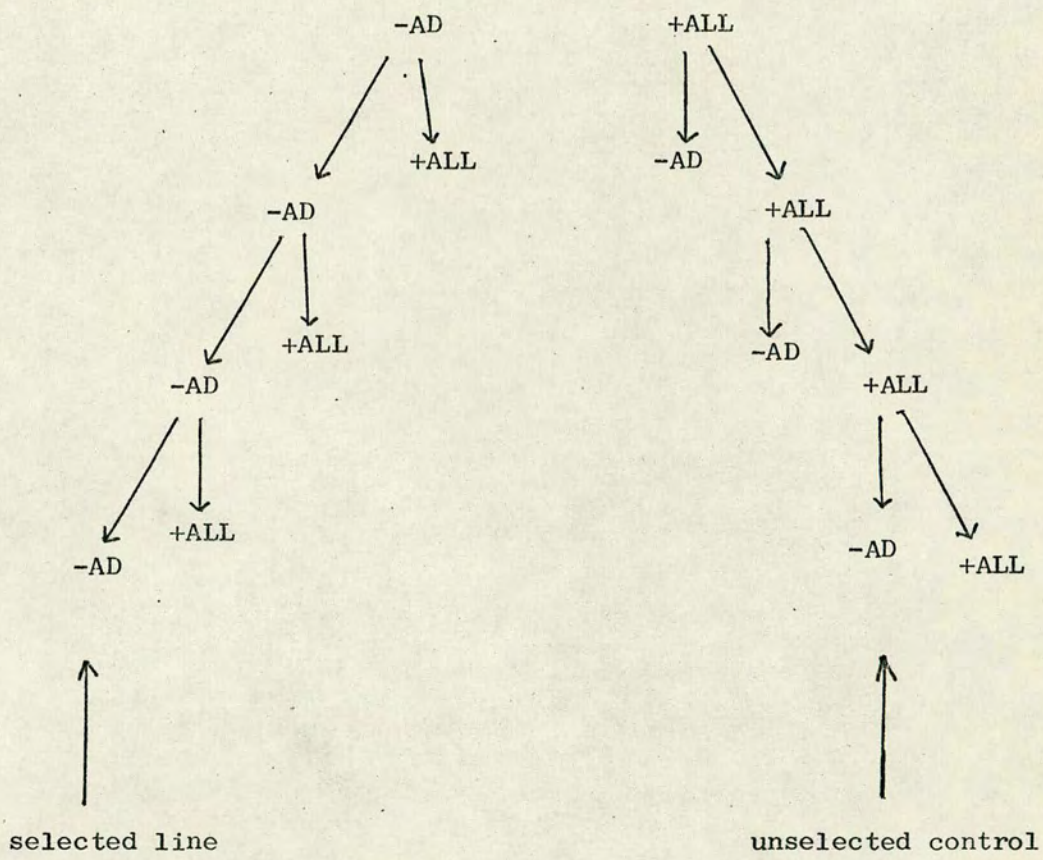
Experiments designed to determine whether, and to what extent, selection for growth on -AD can occur, are reported below.

b) Selection for Growth on -AD Plates

Selection for growth on -AD plates was performed as depicted in Fig. 1. A representative portion of growth of a -AD streak was restreaked onto -AD and +ALL. The latter served as a control to demonstrate the presence of viable cells among those selected. A sample of cells from the original +ALL plate was also restreaked onto -AD and +ALL. Growth on -AD was a control for the selected -AD line and showed the extent of variation possible by chance alone, while the +ALL plate was used to obtain the subsequent -AD control streak. In most cases, selection from -AD was performed as soon as sufficient growth was present for successful transfer. Every effort was made to ensure that equal quantities of yeast were streaked onto each plate.

In these first experiments, 6 haploid and 9 diploid suppressed strains of differing growth ability on -AD were tested. Subsequently, many more strains were shown to exhibit similar behaviour.

Fig. 1 Selection for -AD Growth Ability



A typical selection experiment for a suppressed 69/1/3 isolate progressed as follows: the initial +ALL streak showed good growth after 1-2 days; this was used to initiate the unselected line. The first -AD streak showed slight growth at both ends after 4 days; this was used to initiate the selected line. All subsequent restreaks onto +ALL also grew well, being confluent after 2 days. Control -AD streaks grew more or less to the same extent as the initial -AD streak. Only -AD streaks of the selected line changed in phenotype. The first selected -AD restreak grew marginally better than its unselected control in that end-of-streak growth was apparent after 3 days, while after 4 days slight growth could also be seen in other areas of the streak. On the second -AD restreak, end-of-streak growth was visible after only one day. On the third -AD restreak, intermittant growth along the streak was seen after one day; after two days, almost confluent growth along the streak was seen. After two further restreaks, growth of the selected line on -AD was not significantly different from that on +ALL.

The above experiment took fewer transfers for a good growth phenotype on -AD to be accomplished than did many others involving different suppressed strains. The number of -AD transfers necessary varied a great deal between isolates, and depended to some extent on initial -AD growth capacity. Thus diploid strains, which tended to grow at first less well on -AD than haploid strains, also needed more -AD transfers before good -AD growth was attained. Variation in the time course of selection was also seen, but to a lesser extent, between different selected lines of the same isolate. This was to

be expected in view of the somewhat arbitrary sampling of cells from the -AD streak used for transfer. Assuming variation in growth capacity between individual cells, the cell population transferred was expected to vary in composition between different streaks by chance alone.

That selection acted not on -AD growth ability per se, but on -AD growth rate was indicated by the fact that, if kept for a sufficient length of time (usually at $4^{o}C$ to retard the drying up of the medium), most unselected streaks on -AD eventually grew along the streak, even if initial growth after one week was confined to the ends of the streak. This later growth took weeks or even months to appear as might be expected at low temperatures.

Plates 2a,b, 3 and 4 illustrate selected and unselected lines of 2 suppressed haploid isolates and Plates 5a,b and 6 selected and unselected lines of a suppressed diploid isolate (see legends for details).

No case has been found where selection for good growth on -AD was ineffective. It was concluded that selection for initial growth rate on -AD was possible for all suppressed isolates derived from 69/1. Selection could change an essentially ad^{-} phenotype into an ad^{+} phenotype.

c) Loss of the Selection Effect

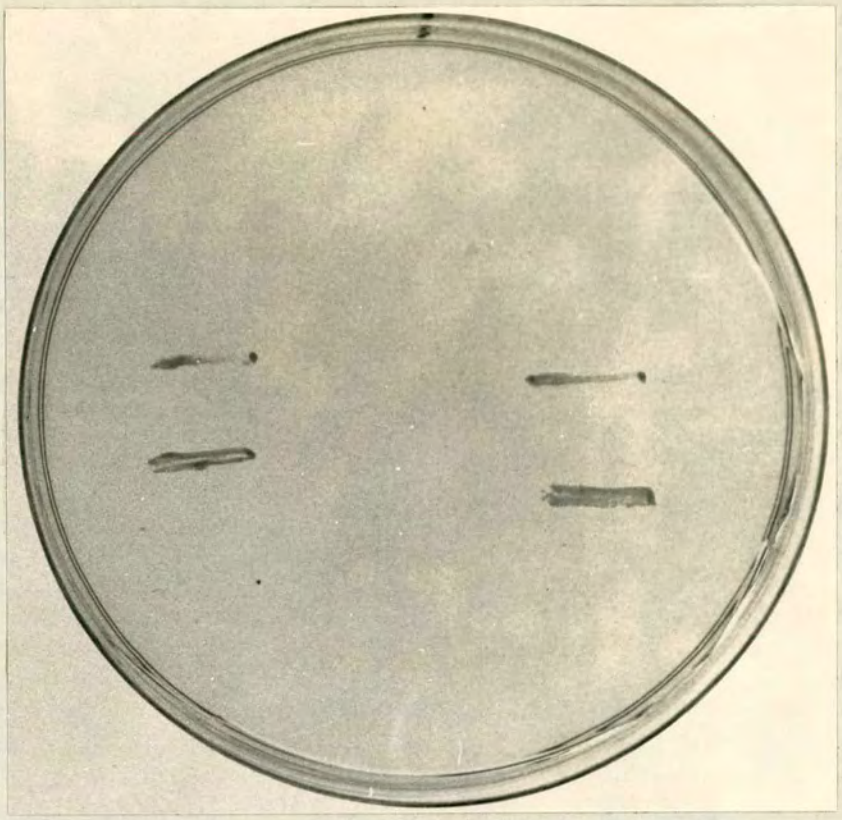
Progressive increase in -AD growth ability may have occurred via an accumulation of modifying mutations acting directly on the adenine biosynthetic pathway or indirectly on the suppression mechanism. If such were the case, the effect of selection would be expected to

Plate 2 Growth of Unselected and Selected lines of Two Suppressed Haploid Isolates

Plates were incubated for 4 days

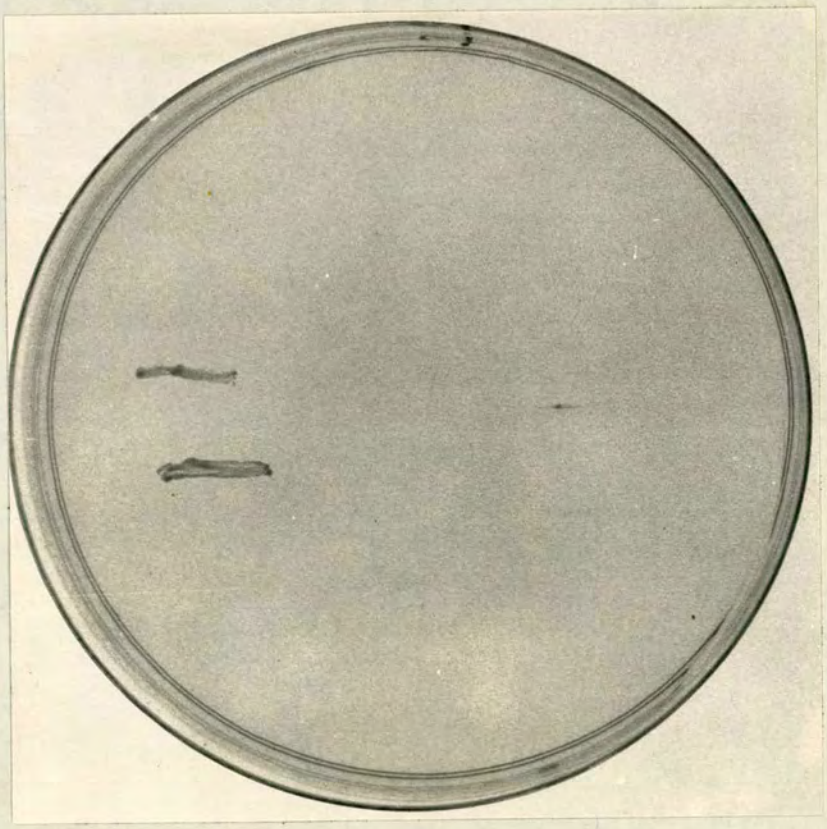
Photographs are approximately life-size

Plate 2



a. Growth on +ALL

2 selected lines streaked on left-hand side of plate and, corresponding unselected controls on right-hand side. All streaks grow



b. Growth on -AD

2 selected lines streaked on left-hand side grow; unselected lines fail to grow.

Plate 3 **Effect of Prolonged Incubation on -AD Growth of Unselected and Selected Lines of Two Suppressed Haploid Isolates**

Plate was incubated for 2 weeks

Photograph is approximately life-size

Plate 3

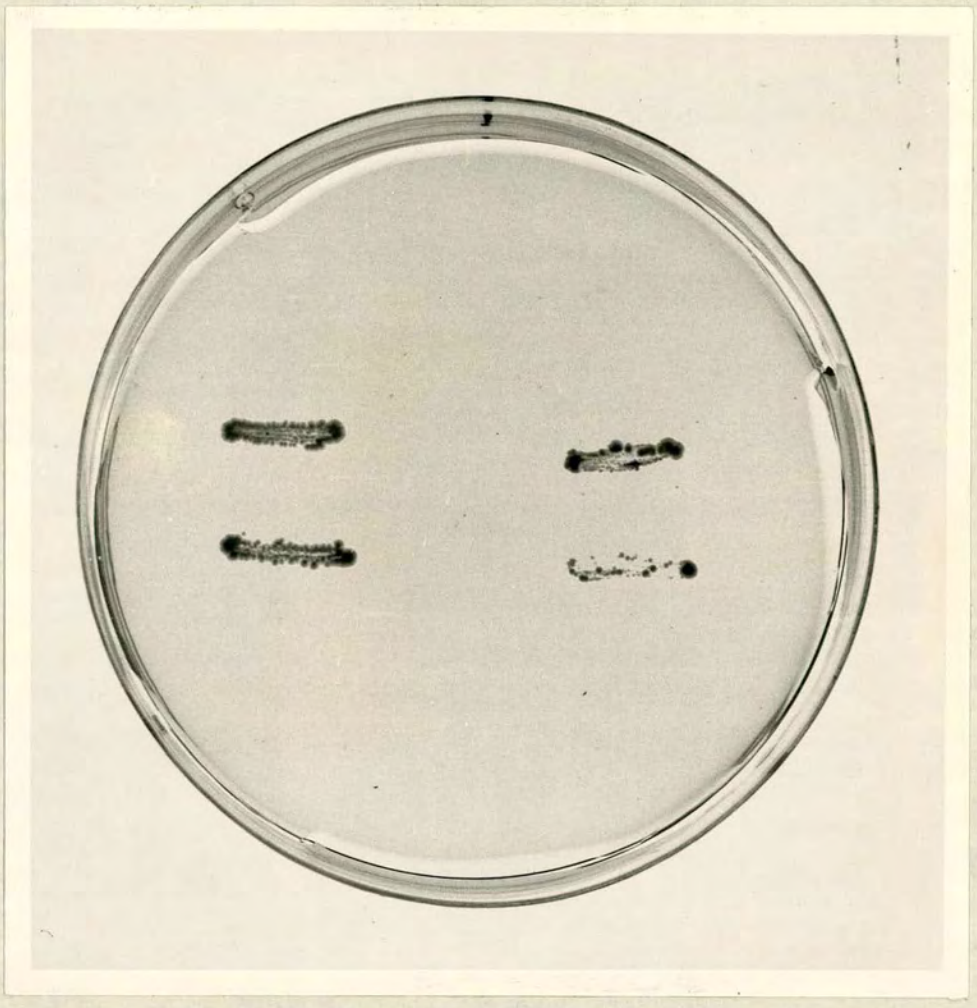
Streaks of unselected lines on right-hand side begin to produce growth

Plate 4 **Effect of Four Weeks Storage on -AD Growth of Unselected and Selected Lines of Two Suppressed Haploid Isolates**

Plate stored at $4^{o}C$ after incubation for 2 weeks

Photograph is approximately life-size.

Plate 4



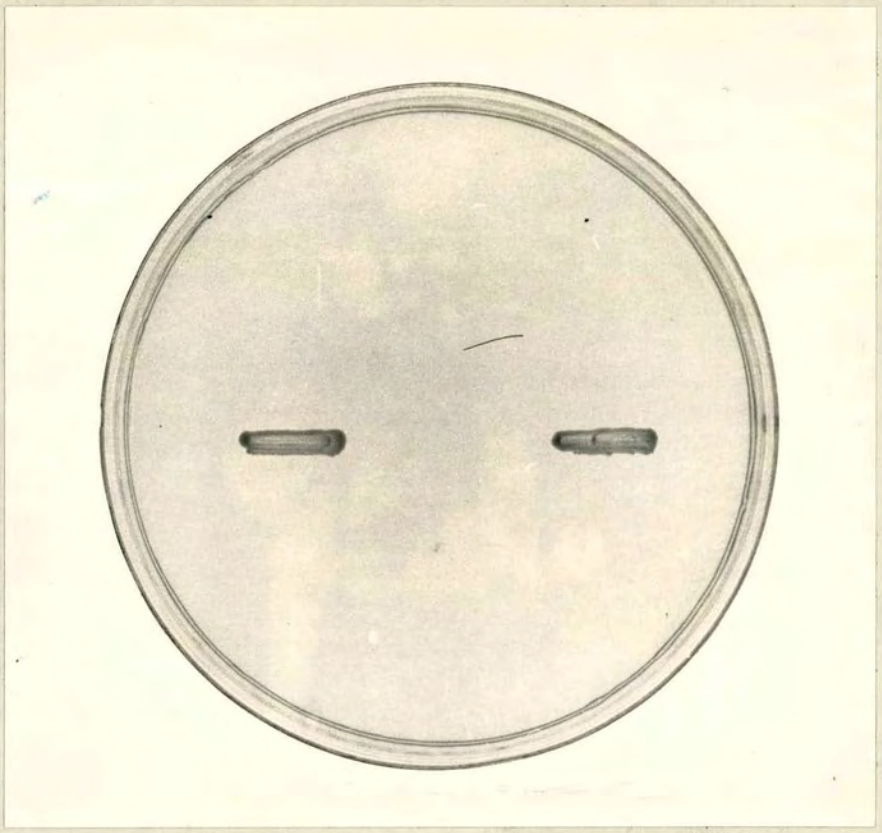
Streaks of unselected lines on right-hand side produce intermittant or almost confluent growth

Plate 5 Growth of Unselected and Selected Lines of a Suppressed 4c Isolate

Plates were incubated for 4 days

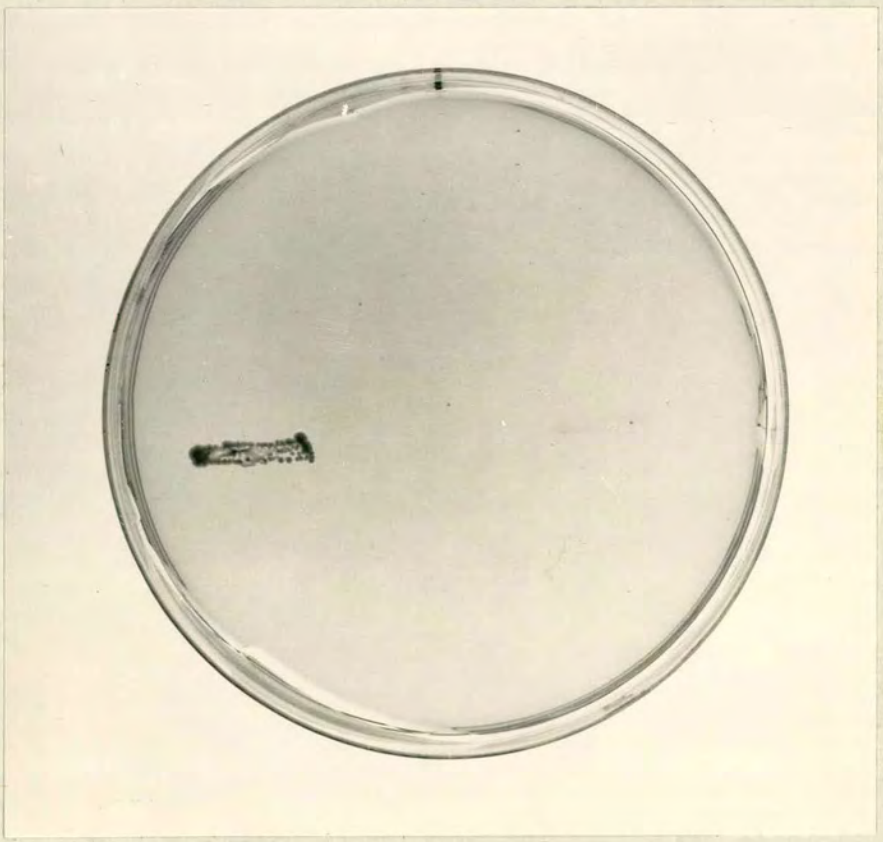
Photographs are approximately life-size

Plate 5



a. Growth on +ALL

Selected line streaked on left-hand side of plate, and unselected line on right-hand side



b. Growth on -AD

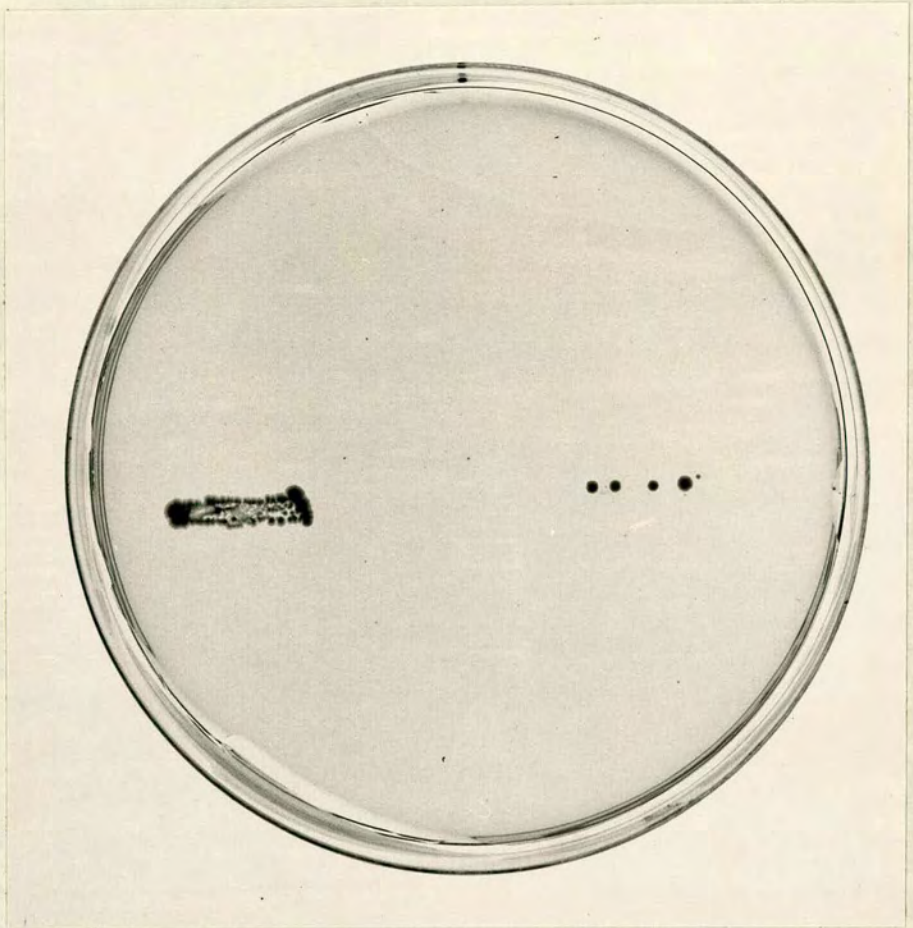
Only selected streak grows

Plate 6 **Effect of Prolonged Incubation on -AD Growth of Unselected and Selected Lines of a Suppressed 4c Isolate**

Plate was incubated for 18 days

Photograph is approximately life-size

Plate 6



4 discrete colonies have appeared on unselected streak

be stable during vegetative growth, and possibly retained to some degree through meiosis. These predictions were tested as follows:

i) Relaxation of Selection for Growth on -AD Plates

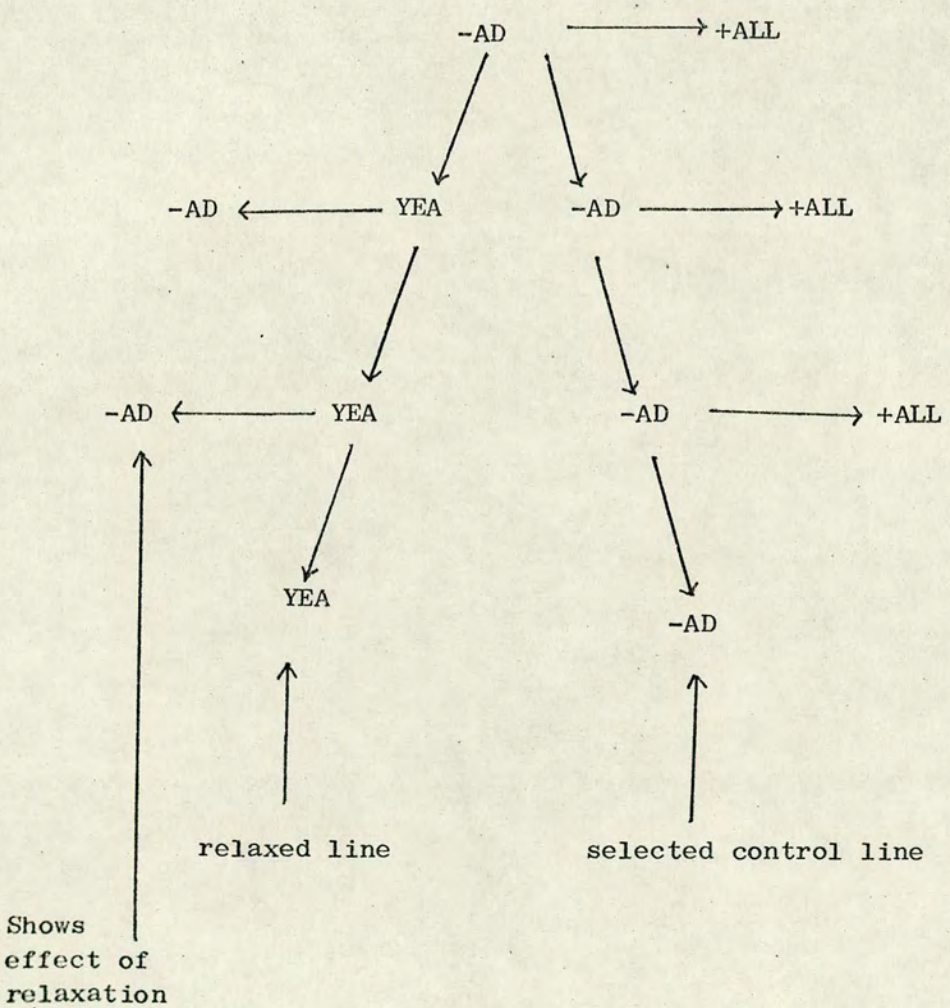
Two isolates selected for -AD growth were used in initial experiments; subsequently, many more were shown to respond in the same manner. The experimental procedure is depicted in Fig. 2.

YEA was used as the non-selective medium. Transfer of both the "deselected" (or "relaxed selection") line and the selected line were made every day. Just as selection had caused a gradual increase in -AD growth ability, it was found that growth on YEA gradually led to the re-appearance of the original growth phenotype on -AD. Both isolates had reverted to the original phenotype after about 5 YEA passages. Subsequent experiments revealed that the time course of the experiment varied between isolates and suppressors, but in every case studied the original phenotype eventually re-appeared on -AD. Again, sampling effects may account for variations found within certain isolates.

This gradual loss of the effect of selection was difficult to reconcile with the hypothesis that increased growth ability on -AD was due to accumulation of nuclear modifying mutations. It would have to be argued that a substantial proportion of cells in each selected isolate retained the original, unmutated genotype, and that these cells grew faster than selected cells on nonselective media. There was, however, no evidence that selected isolates grew more slowly than their unselected controls on +ALL or YEA.

It was concluded that selection probably affected some variable

Fig. 2 Relaxation of Selection for -AD Growth Ability



component(s) of cell metabolism. -AD selection may have imposed a "stress" on the cell system of suppressed strains, which resulted in the "adaptation" of some cytoplasmic factor, thereby increasing adenine-independence. On relaxation of selection pressure, such adaptation would no longer be necessary and may even be detrimental to growth under non-selective conditions. Homeostatic mechanisms would ensure that the cell system returned to the normal, pre-selection state.

ii) Effects of Diploidization and Sporulation

1. Sporulation of Selected $\frac{su_D}{+}$ Strains

4 selected diploids and their unselected controls were transferred to YEA for 2 days presporulation growth, and then to PA to induce sporulation. In all 4 unselected diploids the sporulation frequency was high and the asci appeared to be of normal proportions. All 4 selected diploids, however, sporulated very poorly indeed, the strain with the highest sporulation frequency having a mere 16 asci among the streaks on 5 dissection plates. Asci of selected strains were of abnormal appearance, being smaller and more elongated than normal asci. All 8 diploids were dissected, the number of asci of selected diploids dissected naturally being very limited. Asci of selected diploids also germinated poorly. All ascospore clones were tested for nutritional requirements.

All 4 unselected diploids segregated as expected assuming the presence of a heterozygous super-suppressor. The first selected diploid segregated $4^+:0^-$ for all nutritional markers in each of the 7 tetrads tested. This segregation could most readily be interpreted

in terms of a homozygous super-suppressor, which may have arisen via mitotic conversion at the super-suppressor locus. (Unfortunately, this strain was lost before it could be tested for relaxation of selection on plates). Homozygosity of the suppressor may be one mechanism of attaining better -AD growth, and may account for the selective effect in some diploids. Alternatively, it may have been merely a co-incidental occurence. Clearly, such a mechanism cannot account for the selective effect in haploids.

The second selected diploid yielded only 11 viable ascospores, most derived from different asci. No segregations could be determined, but all haploids appeared to be suppressed.

The third selected diploid yielded only 2 asci, each with 2 viable spores. Both suppressed and unsuppressed spores were present.

The 5 tetrads of the 4th selected diploid segregated as expected assuming the presence of a heterozygous suppressor.

There was no significant difference in growth ability on -AD between progeny of unselected and selected diploids. It was concluded that the effect of selection does not last through meiosis, and is therefore unlikely to involve mutation at one or a small number of loci. More probably, cytoplasmic changes, abolished during the molecular rearrangements required for meiosis, were involved. In some cases, homozygosity of the suppressor locus may be the mechanism upon which selection acts. Selection caused reduced sporulation frequency and abnormally-shaped asci to be formed.

Two factors demand that only tentative interpretations of these

results be made. First, the extremely low sporulation frequency of the selected diploids may imply that those asci which did occur are unrepresentative of the diploid. Second, growth on non-selective media was unavoidable during sporulation induction. Loss of the selection effect may have occurred for this reason rather than as a result of meiosis itself.

2. Diploidization of Selected su_{II} Strains

Whether selection occurred via genic or cytoplasmic changes, it was considered of interest to enquire whether increased -AD growth in haploids is dominant or recessive.

A selected derivative of 69/1/3 and its unselected parent were each crossed to unsuppressed 69/1/9, using the YEA mass mating technique since no forcing markers were present. The unselected haploid crossed well with 69/1/9; 3 diploids, probably of independent origin, were isolated and all sporulated at high frequency when tested for diploidy on PA. A very low sporulation frequency was seen on the PA plate of mass mated 69/1/9 and the selected haploid. This may indicate low crossing ability of the selected haploid or low sporulation ability of the diploids formed. Only one of the 10 single colonies isolated from the mass mating could be classified as diploid by the PA test. It displayed a fairly low sporulation frequency. The diploids formed were tested on -AD, -ARG and -LYS. All grew well on -ARG and -LYS, but very poorly on -AD.

It was concluded that the selective effect was, in effect, recessive in the diploid. Selection adversely affected the ability to undergo diploidization and/or sporulation. Again, loss of the selective effect may have been caused by growth on YEA, a non-selective

medium, prior to testing on -AD, perhaps only those haploids which had lost the effects of selection forming diploids.

iii) Summary

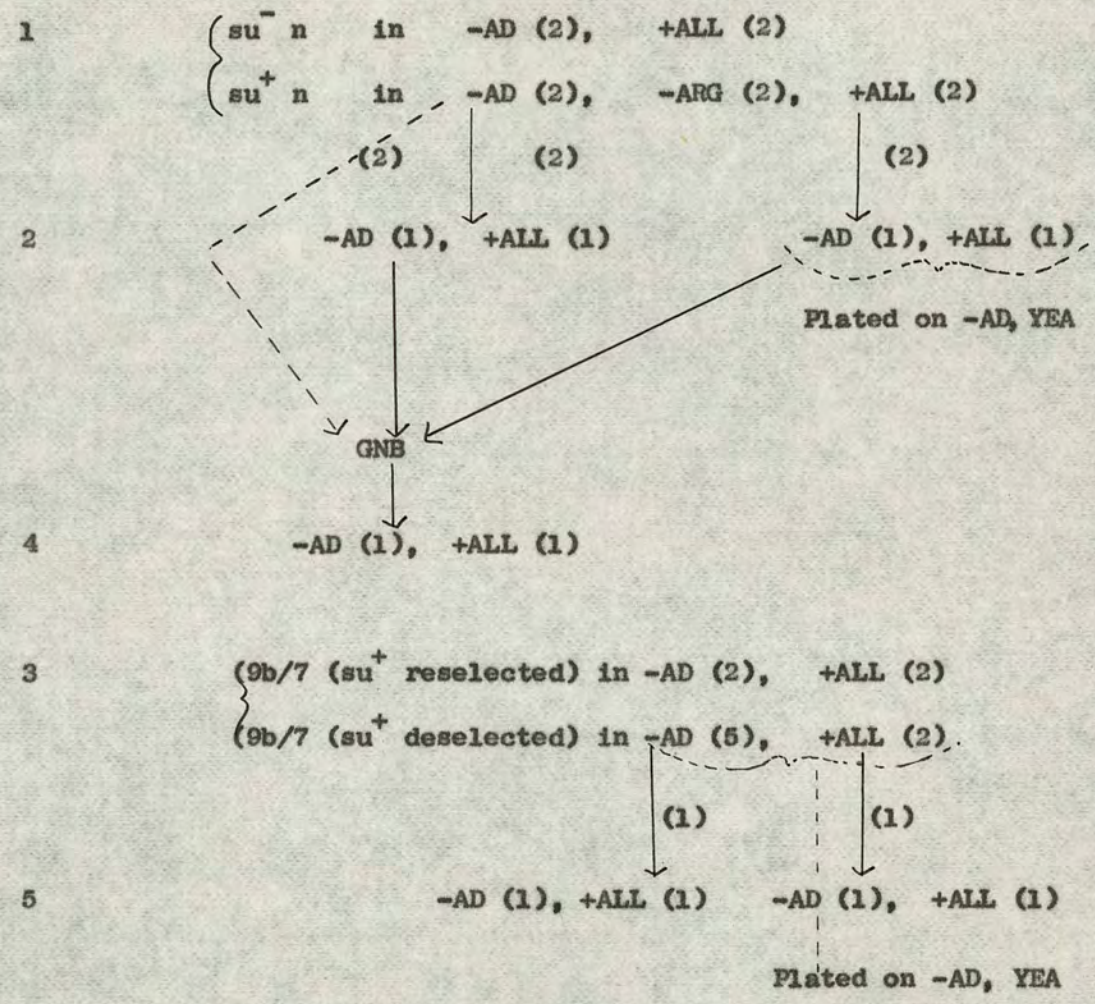
The selective effect described appeared to be unstable; it was lost on relaxation of selection pressure and possibly also on changes of ploidy. These characteristics implicated cytoplasmic rather than genic changes as those upon which selection acted. Selection adversely affected sporulation and possibly also diploidization processes.

d) Growth Experiments Using Liquid Media

In order to quantify the effect of selection on -AD growth, experiments using liquid growth media were performed. It was hoped that growth curves might provide information on the mechanism involved in selection. The relationships between some of the various Liquid Growth Experiments to be described are shown in Fig. 3.

The same basic procedure was followed in all the subsequent growth experiments. This was as follows: a suspension of the appropriate strain at 5×10^5 cells/ml citrate buffer was made up. 1 ml of this suspension was pipetted into 100 ml relevant medium in a 250 ml flask. Appropriate dilutions of this suspension were immediately plated on YEA. Flasks were shaken at $32^{\circ}C$. After various time intervals, the concentration of cells was determined by means of a haemocytometer and suitable dilutions plated on YEA. Citrate buffer was used for dilution to minimize error due to the "clumping" of cells, often a problem when using liquid media.

Fig. 3 Relationships Between Liquid Growth Experiments 1-5



( ) = number of flasks used

YEA plates were scored after 2-3 days incubation unless otherwise stated.

i) Liquid Growth Experiment 1: Growth Curves of Suppressed and Unsuppressed Haploid Strains

Su^- (unsuppressed) and su^+ (suppressed) haploids with strictly isogenic backgrounds were unavailable when this initial experiment was carried out. Therefore, su^- and su^+ ascospore clones from the same dissected tetrad of a suppressed 4c strain, expected to have very similar genetic backgrounds, were used. Su^+ growth on -AD plates was better than that usually found in the absence of selection, being almost confluent after about 3 days.

Suspensions of each strain were made from +ALL streaks. 2 flasks each of +ALL and -AD were used for each strain. In addition, 2 flasks of -ARG were used for su^+. The results are shown in Figs. 4a,b, which are plots of haemocytometer and plate counts respectively. Replicate flasks gave almost identical results in all cases except that of su^+ in -AD. Only for the latter, therefore, are results for individual flasks shown.

Figs. 4a,b show:

1) The 2 sets of curves show good correspondence. Haemocytometer count was concluded to give a reliable estimate of cell concentration. After the onset of stationary phase, cell concentration remained constant while the viable cell count declined.

2) Su^- in +ALL attained a lower stationary phase cell concentration than did su^+ cultures, and also showed a more rapid decline in viable cell concentration, after the exponential growth phase, than any su^+ culture.

Fig. 4a Liquid Growth Experiment 1 (Haemocytometer Counts)

- ● su^+ in +ALL (2 replicates, (1) and (2), virtually identical)
- ■ su^+ in -ARG (ditto)
- ▲ su^+ in -AD (1)
- ▼ su^+ in -AD (2)
- ○ su^- in +ALL

Fig. 4a

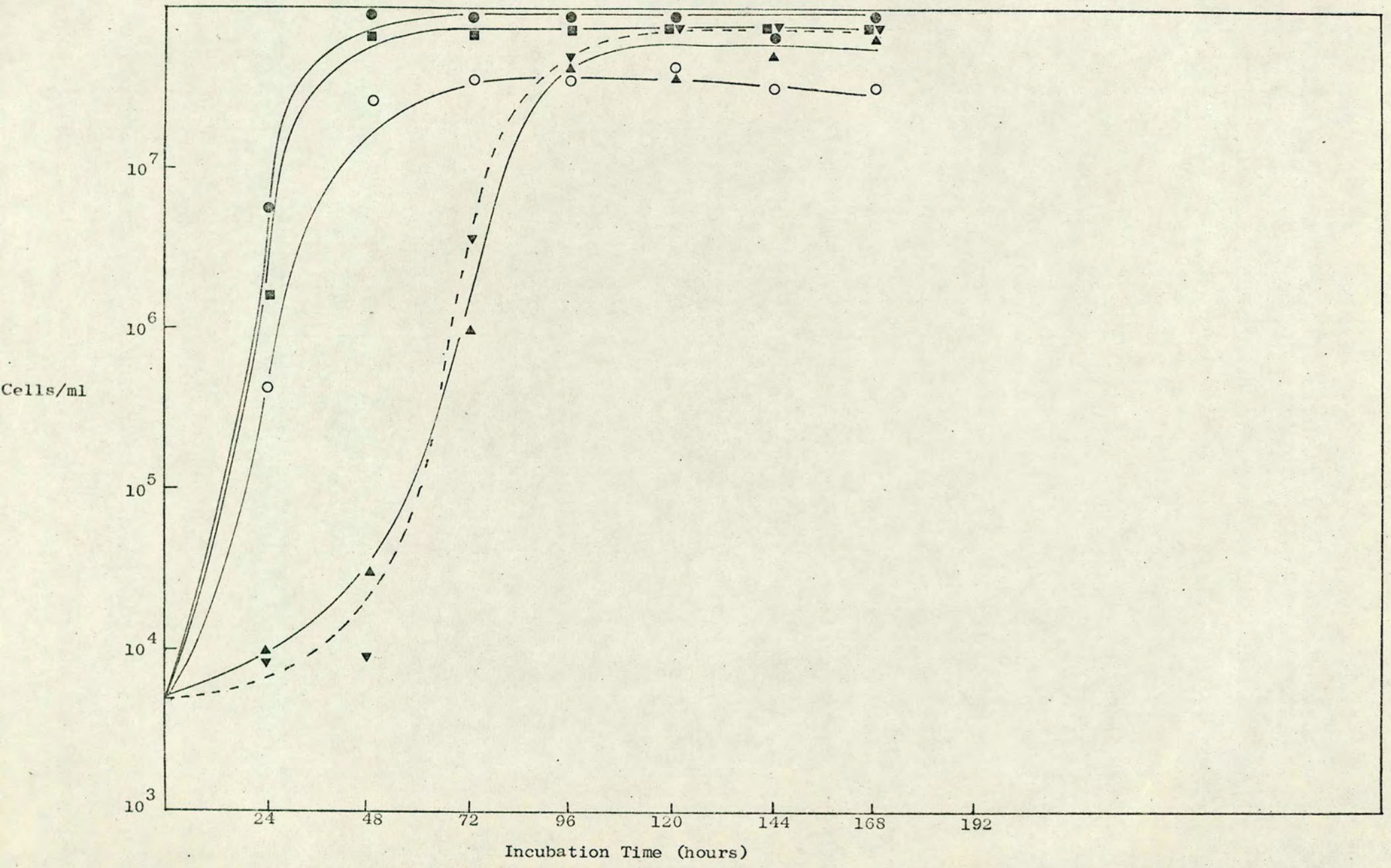

Cells/ml
10^7
10^6
10^5
10^4
10^3
24
48
72
96
120
144
168
192
Incubation Time (hours)

Fig. 4b Liquid Growth Experiment 1 (Plate counts)

● su^+ in +ALL (2 replicates, (1) and (2), virtually identical)

■ su^+ in -ARG (ditto)

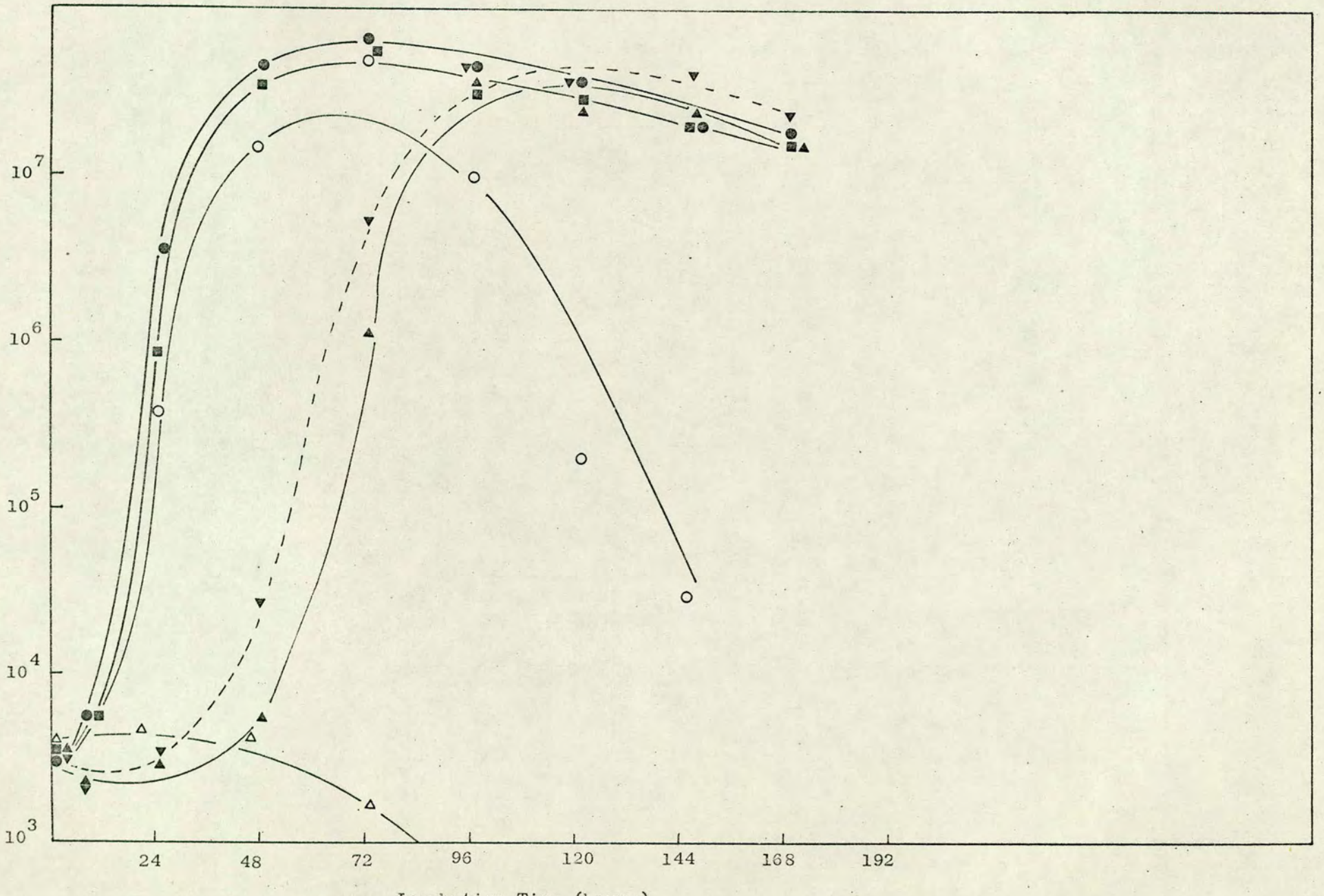
▲ su^+ in -AD (1)

▼ su^+ in -AD (2)

△ su^- in -AD (2 replicates virtually identical)

○ su^- in +ALL (ditto)

Fig. 4b


10^7
10^6
10^5
10^4
10^3
Cells/ml
24
48
72
96
120
144
168
192
Incubation Time (hours)

3) Growth curves of su^+ in +ALL and -ARG were almost identical.

4) After a lag of 24-48 hours, su^+ grew in -AD at approximately the same growth rate (as judged by the slope of the exponential portion of the curve) as initially found in +ALL. Su^- did not grow in -AD.

Su^+ growth in -AD was interesting for the initial lag phase. Such a curve may represent an initial "resting" period during which no cell division takes place, followed by division of the whole cell population at approximately the same growth rate as that in +ALL medium. Alternatively, the growth observed may originate from a very small base population able to grow in -AD at normal growth rates. Growth of such a population, in the presence of about 5×10^3 non-dividing cells per ml, would result in a sigmoid growth curve. This can be seen by reference to Fig. 5. Fig. 5 illustrates theoretical growth curves, assuming a 3 hour doubling time, for various initial concentrations of viable cells. Also shown is the resultant curve obtained if curve 'c' were superimposed upon 5×10^3 cells/ml of a viable but non-dividing culture. The resultant curve is very similar to that of su^+ in -AD in Figs. 4a,b.

If division of the whole population occurs, a lag period might be required for the synthesis of a sufficient quantity of some specific product, or for the "adaptation" of certain metabolic processes; only when this had been achieved could cell division take place.

A true $ad_{2.1}$ revertant, an additional super-suppressor mutation or a "pre-adapted" cell (i.e. one whose metabolic state was already

Fig. 5 Growth Curves Expected to be Generated by Various Initial Cell Concentrations Assuming a 3 Hour Doubling Time

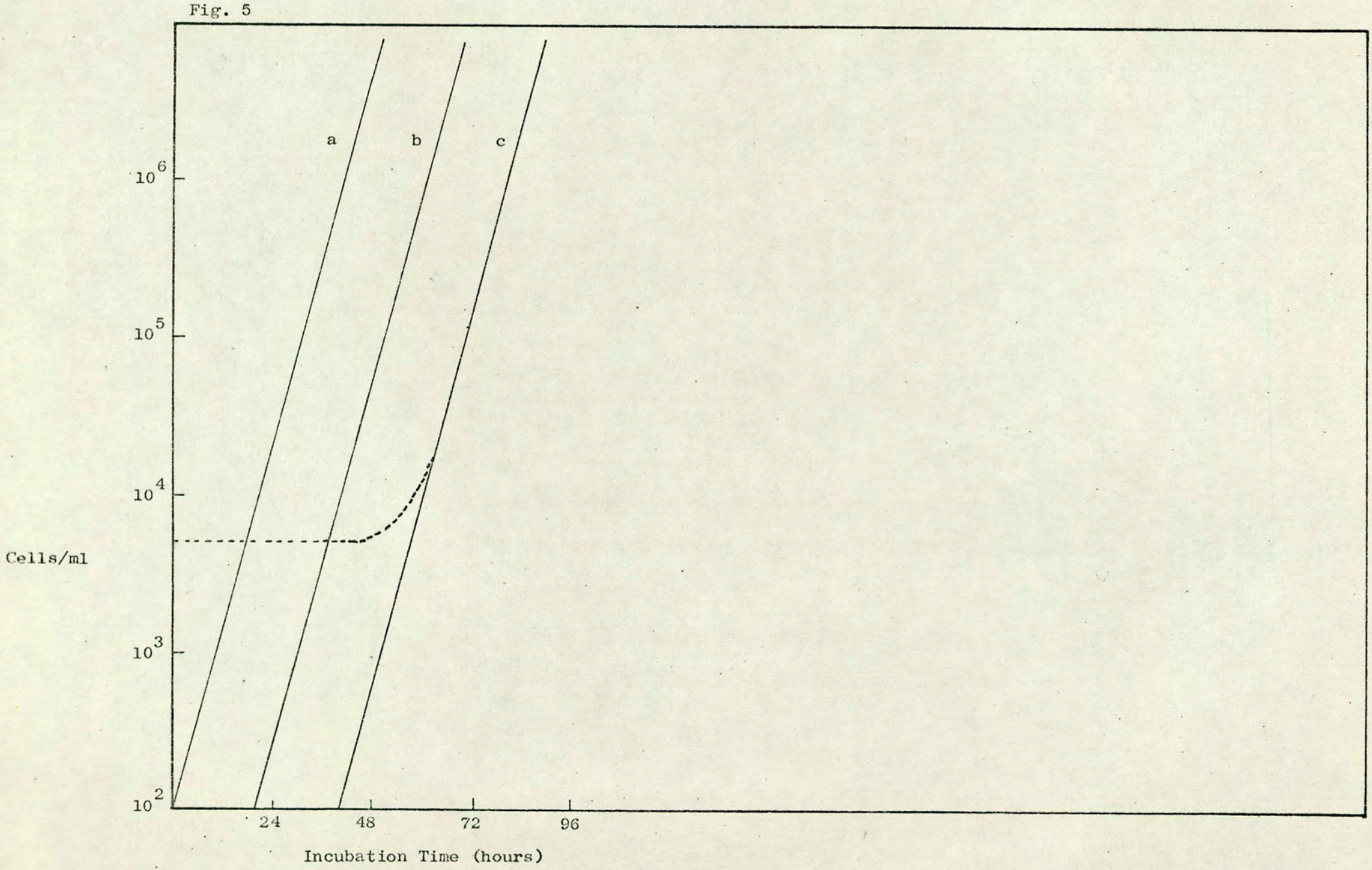
a : 10,000 cells inoculated (i.e. concentration = 100 cells/ml)

b : 100 cells inoculated (i.e. concentration = 1 cell/ml)

c : 1 cell inoculated (i.e. concentration = 0.01 cells/ml)

_ _ _ _ _ : resultant curve expected if 5×10^3 viable cells, incapable of division in liquid medium, are present in addition to one cell capable of such division.

Fig. 5


a
b
c
10^6
10^5
10^4
10^3
10^2
Cells/ml
24
48
72
96
Incubation Time (hours)

compatible with -AD growth) might have given rise to a small initial population which divided immediately upon inoculation into -AD liquid medium. Growth to stationary phase of such a population might have occurred before significant division of the majority of inoculated cells could take place. If mutated cells were responsible for growth, the stationary phase culture would have consisted of a genotypically different population from that inoculated.

Whatever the mechanism responsible for -AD growth, it was considered important to determine the growth characteristics of the su^+ strain upon re-inoculation into fresh -AD liquid medium.

ii) Liquid Growth Experiment 2: Effect of Pregrowth in -AD on Subsequent -AD Growth

168-hour su^+ cultures of Liquid Growth Experiment 1 were used for sub-inoculation into fresh media. Appropriate aliquots from both -AD and +ALL flasks, containing approximately 5×10^5 viable cells, were used to inoculate one flask each of fresh -AD and +ALL (8 flasks in all). The growth curves obtained are shown in Fig. 6.

It can be seen that the pattern of growth in each +ALL flask was very similar to that found in Experiment 1. Also, both -AD flasks inoculated with cells pregrown in +ALL showed the 24-48 hour lag found for -AD flasks in Experiment 1. However, both inocula from -AD grew in fresh -AD with no lag, but with a curve very similar to that obtained from +ALL.

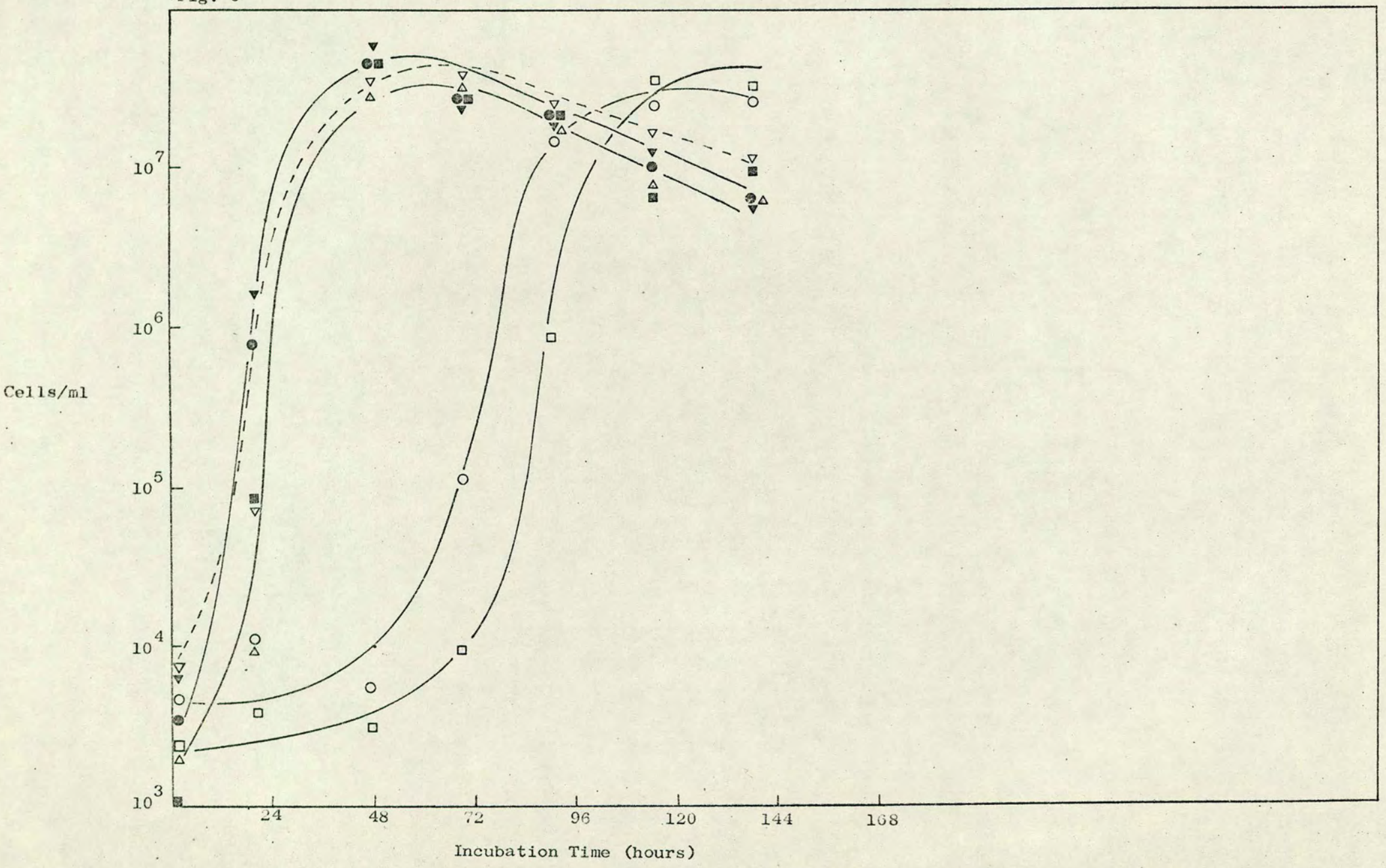
It was inferred that pregrowth in -AD in some way altered the cell population such that it was better able to grow on subsequent re-inoculation into fresh -AD medium. This "alteration" may have

Fig. 6 **Liquid Growth Experiment 2**

●	+ALL (1) and (2)	into +ALL (2 curves virtually identical)
■	-AD (1)	into +ALL
▼	-AD (2)	into +ALL
○	+ALL (1)	into -AD (a)
□	+ALL (2)	into -AD (b)
△	-AD (1)	into -AD (c)
▽	-AD (2)	into -AD (d)

All inocula were derived from Liquid Growth Experiment 1 cultures (see Fig. 4b).

Fig. 6


10^7
10^6
10^5
10^4
10^3
Cells/ml
24
48
72
96
120
144
168
Incubation Time (hours)

occurred via a mechanism involving cell selection, adaptation or a combination of these two. Selection of true ad^+ revertants or additional su^+ mutations was considered unlikely as no such mutations had been recovered in previous experiments using solid medium, even those in which selective pressure for -AD growth ability was applied. Section iii) below describes experiments which eliminated the possibility of mutant selection altogether.

iii) Experiments Designed to Distinguish between Mechanisms Involving Mutation and Adaptation

1. Outcrossing of Cells Grown in -AD

If a true ad^+ revertant was selected in Experiment 1 -AD flasks, then on crossing it to a strain wild-type at the AD 2 locus and sporulating the resulting diploid, no ad^- progeny should result.

Su^+ cells from both +ALL and -AD cultures of Experiment 1 were crossed to an ad_2^+ $arg_{4.19}$ haploid. The diploids formed were sporulated. Colonies from random ascospore plates were tested on -AD plates. Both types of diploid yielded ad^- progeny. It was concluded that an ad_2^+ mutant was not responsible for -AD growth in Experiment 1.

2. Liquid Growth Experiment 3: Correlation of Growth on -AD Plates with Lag in -AD Liquid Medium

The su^+ strain used in Experiments 1 and 2 grew on -AD plates within 2-3 days. If the lag in -AD was caused by the necessity for cells to adapt to this medium, a relationship might be expected between growth capacity on -AD plates and the duration of the lag period in -AD liquid. This was explored by using suppressed haploid isolates which grew very slowly on -AD plates.

Suppressed isolates of the 69/1/3-derived multiply-marked haploid strain, 96/7, grew very poorly on -AD plates (showing only sparse growth on -AD after about 10 days). Selection on plates for -AD growth had previously been successfully carried out for a suppressed 96/7 isolate. A relaxed selection line (displaying the pre-selection growth phenotype on -AD) of this isolate was available. Reselection of this line was carried out. Confluent growth on -AD after 2 days was eventually established. Growth curves of both deselected and reselected lines were obtained in Experiment 3 (using growth on +ALL and -AD plates respectively for making up inocula). The results are shown in Figs. 7a,b.

The deselected strain grew in -AD after a minimum lag of approximately 130 hours. The reselected strain showed no such lag. The growth rate of the latter in both -AD and +ALL was lower (as judged by the slope of the curve during exponential growth) than that of the deselected strain, a property reflected in growth on YEA plates: those of the deselected strain could be scored after 2 days, whereas those of the reselected strain could be counted only after 3 days.

The deselected strain exhibited different lag periods in each of the 5 -AD flasks used. No growth at all was obtained in one of the flasks, while the remaining 4 supported growth after lags ranging from about 130-150 hours.

It appeared that a definite correlation existed between growth ability on -AD plates and length of lag period in -AD liquid medium, viz: the faster the growth on -AD plates, the shorter the lag period

Fig. 7a Liquid Growth Experiment 3 (Deselected Line)

● +ALL (2 replicates, a and b, virtually identical)

△ -AD (1)

□ -AD (2)

○ -AD (3)

▽ -AD (4)

-AD (5) failed to grow

Fig. 7a

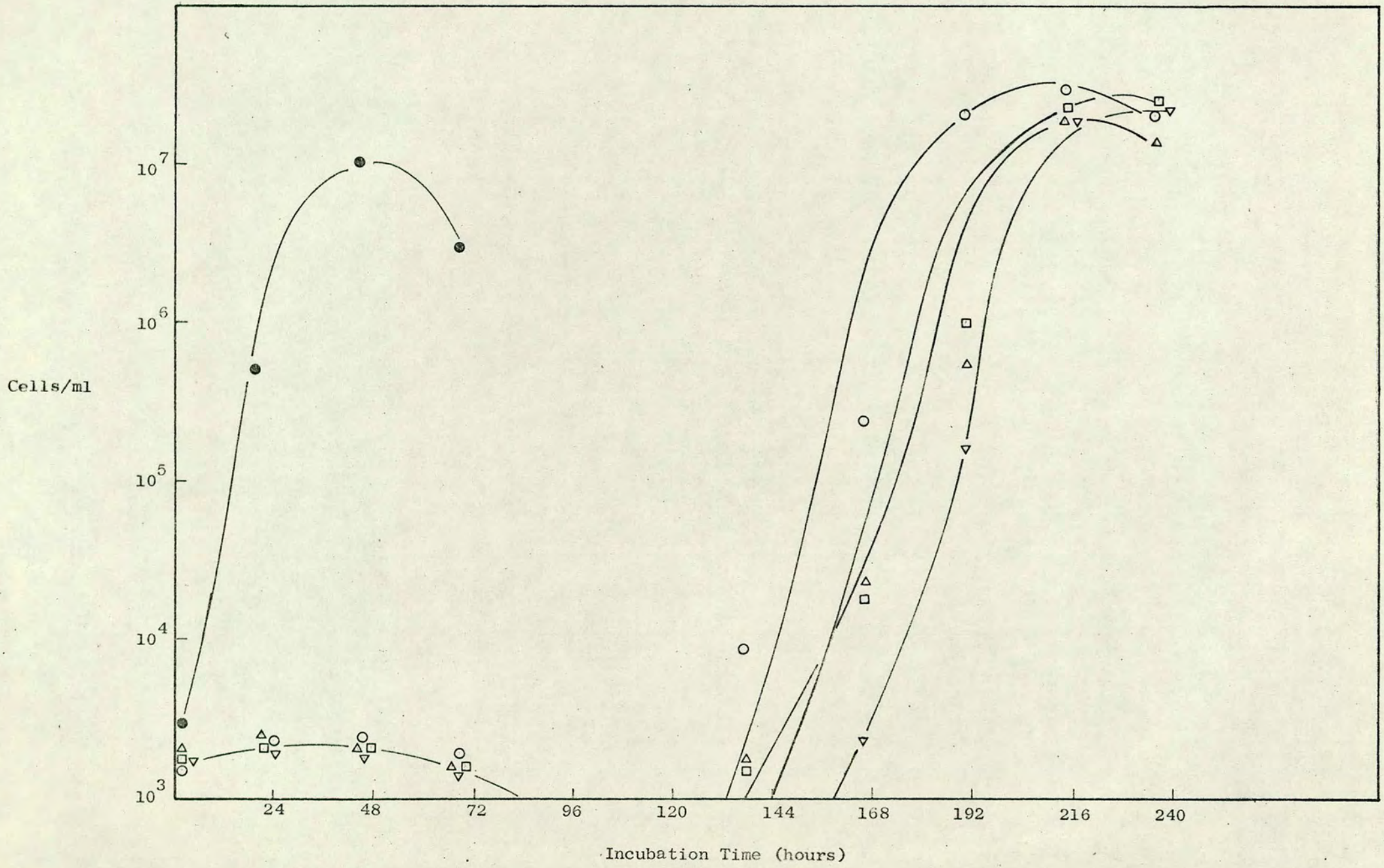

Cells/ml
10^7
10^6
10^5
10^4
10^3
24
48
72
96
120
144
168
192
216
240
Incubation Time (hours)

Fig. 7b Liquid Growth Experiment 3 (Reselected Line)

○ +ALL (2 replicates virtually identical)

△ -AD (ditto)

in -AD liquid medium. (This correlation was later confirmed in experiments using suppressed diploids, reported below).

Growth of suppressed strains on -AD plates was previously concluded to be explicable in terms of weak suppression rather than mutation to ad^+ colonies (see Section I d)). That a correlation exists between suppressed growth ability on -AD solid medium and frequency of ad^+ or su^+ mutations in -AD liquid medium (as measured by the length of the lag) seemed highly improbable. Such mutations were expected to occur at random (initiating growth at any time from only a few hours to perhaps several days after inoculation) and to show no correlation with penetrance of suppressors on -AD plates. That growth ability on -AD plates is related in some way to that in -AD liquid medium, possibly via some "adaptation" mechanism was considered more feasible. This topic will be discussed below.

3. Liquid Growth Experiment 4: Re-introduction of the Lag Period

Growth in -AD prior to re-inoculation into fresh -AD liquid medium eliminated the lag period characteristic of initial -AD growth. If the lag can be re-introduced by growth in complete, non-selective liquid medium, then the initial -AD growth is unlikely to have been attributable to ad^+ or su^+ mutations. Only if a proportion of cells of non-mutant genotype are present in the -AD sub-inoculum and have a selective advantage in complete medium sufficient to out-grow the majority of mutant cells, could elimination of a -AD pregrowth effect due to ad^+ or su^+ mutants occur. There is no evidence that these prerequisites exist: su^- did not grow at a higher rate than su^+ in

in +ALL flasks of Experiment 1; the growth rate of these $ad_{2.1}$ strains in +ALL was approximately 1 division every $2\frac{2}{3}$ hours, not significantly higher than that for ad^+ strains in complete medium. It was therefore reasoned that re-establishment of a growth lag in -AD would be evidence that its previous elimination was not caused by growth of ad^+ or su^+ mutants.

Liquid Growth Experiment 4 was performed to establish whether growth in non-selective medium could eliminate the -AD pregrowth effect. Cultures from 4 -AD flasks were used. These were: both -AD flasks from Experiment 1 and two -AD flasks of Experiment 2, one previously inoculated from +ALL and the other from -AD flasks of Experiment 1. For each culture the procedure was as follows: 1 ml of stationary phase suspension from the -AD flask was inoculated into 30 ml GNB in a 100 ml flask and shaken at $32^{\circ}C$ for 24 hours, after which time a 1 ml aliquot was transferred into fresh GNB. After 9 such transfers, the suspension was spun, washed and a suspension in citrate buffer at 5×10^5 cell/ml prepared. 1 ml of this suspension was inoculated into one flask each of -AD and +ALL. Growth curves were plotted. Results are shown in Figs. 8a,b.

Growth in GNB had no effect on subsequent growth in +ALL, but did have an effect on that in -AD. Fig. 8a shows that one -AD-derived culture displayed the 24-48 hour lag in -AD characteristic of initial growth curves, while the other grew after a lag of 0-24 hours. Fig. 8b shows that the original lag period of -AD cultures not previously exposed to -AD was retained by both -AD-derived cultures by growth in GNB.

Fig. 8a Liquid Growth Experiment 4

● +ALL
○ -AD
} Inocula derived from -AD (1) culture of Liquid Growth Experiment 1

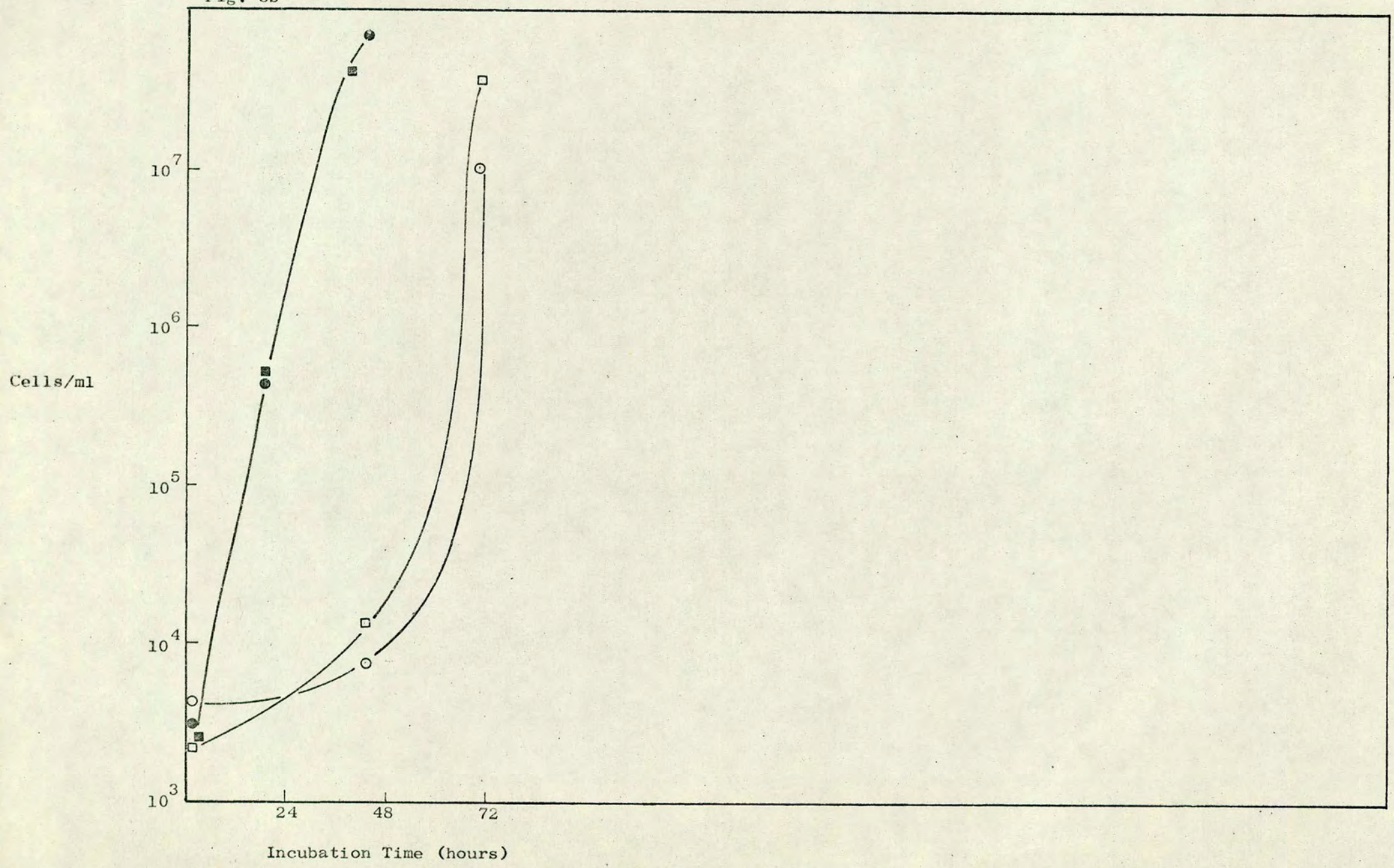
■ +ALL
□ -AD
} Inocula derived from -AD (2) culture of Liquid Growth Experiment 1

Fig. 8b Liquid Growth Experiment 4

● +ALL } Inocula derived from -AD (c) culture of
○ -AD } Liquid Growth Experiment 2 (see Fig. 6)

■ +ALL } Inocula derived from -AD (a) culture of
□ -AD } Liquid Growth Experiment 2 (see Fig. 6)

Fig. 8b



It appeared that growth on non-selective medium did indeed negate the effect of growth in -AD on subsequent -AD growth curves. The process responsible for this loss of -AD pregrowth effect appeared to have gone to completion in 3 of the 4 cases, but to have been only partially successful where the 0-24 hour lag was present.

Again it was concluded that ad^+ or additional su^+ mutants were not responsible for the -AD pregrowth effect.

4. Summary and Discussion

The conclusion that growth in -AD liquid medium in Liquid Growth Experiment 1 was not due to selection of mutant cells was reached. The following observations pointed to the involvement of some sort of adaptive mechanism determining -AD growth curve characteristics:

a) The lag period characteristic of initial -AD growth

b) Absence of the above lag on re-inoculation of -AD cultures into fresh -AD medium.

c) Reappearance of the lag by interruption of the inoculation procedure in b) above by growth in GNB.

d) The lag of approximately 130 hours, inexplicable on the basis of growth arising from a single cell of the inoculum capable of immediate growth at a normal rate.

Observation d) above implies that adaptation must occur within the -AD medium, a lag of 130 hours being too long to be accounted for by introduction of a single pre-adapted cell. In other words, a hypothesis based on the selection of rare mutant cells would also require the assumption of adaptation of such cells to account for

such a lengthy lag phase.

Adaptation of either the whole population or a fraction of it was considered possible. In the former case (where adaptation ability of each individual cell is very similar), all cells of the population would take an approximately equal time to adapt; division of all cells would then occur within a short time. In the latter case (where adaptability of individual cells may be normally distributed), one or a small number of cells would adapt, divide at a normal rate, and outgrow the parent population. Situations between these two extremes are, of course, possible.

The assumption is made that in any population of cells, even those derived from a single cell, no two cells will be identical. All cells of a clone may appear to be alike genotypically and phenotypically, but our assessment of the situation is limited both by the scope and accuracy of the methods we use and by the measurements we choose to make. Inevitably, measurement of some properties will involve changing those very characteristics we are attempting to evaluate. However, it must be intuitively obvious that each cell of a population occupies a unique environment.

Each cell may be thought of as being in dynamic equilibrium with its surroundings. Differences in micro-environment result in differences in cytoplasmic state between individual cells. Change of environmental condition leads to correlated cytoplasmic changes. Because of the complex, interacting nature of living systems, no change can occur in isolation, but will inevitably effect other changes. The dynamics of cellular systems demand that these correl-

ated changes will themselves be "balanced" by yet more changes. Thus a single initial change in environmental condition may, potentially at least, lead to innumerable and unpredictable consequences.

Numerous examples of a change in cellular activity consequent upon an environmental change may be quoted. The following examples are but a few: induction or repression of genes whose products are involved in the metabolism or catabolism of certain metabolites present in the surrounding medium; induction of meiosis in yeast by PA medium; variation in repair capacities in yeast following UV-irradiation dependent on the plating medium employed; variation in red pigment accumulation in ad_1 and ad_2 mutants of yeast depending on factors such as aeration, glucose concentration and variety and amount of purines in the medium.

That each cell occupies a unique environment, hence maintaining a unique cytoplasmic state may be appreciated by consideration of "spontaneous" genetic events. That, for instance, spontaneous mutations occur in some cells but not in others is indisputable; why such mutations occur in one rather than another is not so obvious. However, despite the fact that we may be unable to pinpoint one particular "cause", it must be true that the occurrence of the mutation was an outcome of the activity of a particular cytoplasmic state.

Similarly, such questions as: why do some cells undergo mitotic recombination while others do not?; what determines segregation pattern at meiosis? and so on, may be answered in terms of the differences in physiological state between cells which means that

no two cells are exactly alike.

Results obtained on -AD plates and their correlation with growth lag in -AD liquid medium are consistent with this assumed situation. It is proposed that adaptation (defining "adaptation" as a change in physiological state leading to increased growth ability on a particular medium) occurs within a time dependent on certain physiological factors within a given population. Some physiological states may be more favourable to -AD growth than others and will take less time to adapt. This could explain:

a) The initial variation in growth ability on -AD plates

b) Variation in selection times on -AD plates

c) Variation in lag period for different isolates of the same strain, as for the 5 -AD flasks of Liquid Growth Experiment 3.

d) Variation in time required in non-selective medium to restore the original -AD lag (Experiment 4).

Thus suppressed strains capable of growth on -AD plates within 2-3 days may have a cytoplasmic state already fairly well adapted to -AD growth, and show a lag of only 24-48 hours in liquid -AD medium. Those strains which grow extremely slowly on -AD plates have cytoplasms less well adapted to -AD and show a lag of over 130 hours, as the systems required for -AD growth require a longer exposure time to the selective medium before adaptation is complete. This reasoning applies whether growth originates from the whole population inoculated or from a single cell. In either case, the factors selected by the adaptive mechanism can be considered to be

distributed randomly throughout a population, the mean determining the length of the lag in -AD liquid. The "higher" (towards adaptation) the mean, the sooner will the whole population adapt, or the sooner is any particular cell likely to complete adaptation and grow.

The slopes of the curves during exponential growth phase are approximately equal for suppressed haploids grown in -AD and in +ALL. This suggested that those cells capable of -AD growth are maximally adapted for such growth, a suggestion supported by the lack of -AD lag on re-inoculation from -AD in Experiment 2. Partial adaptation, resulting in a prolonged doubling time, does not appear to occur,or, if it does, to persist. However, selection experiments on -AD plates had previously suggested that adaptation is not a one-step process: restreaking of single colonies did not lead immediately to confluent growth; rather, improvement in -AD growth was achieved progressively.

It should be made clear, however, that comparison between selection for growth on -AD plates and growth in -AD liquid medium, though probably both manifestations of the activity of the system governing response to -AD medium, are probably not equivalent processes and therefore are not expected to exhibit exactly comparable properties. Not only are solid and liquid -AD media different with respect to such properties as availability of nutrients, survival time of non-dividing cells, etc., but also in the following respect: if a single cell of a -AD streak can grow, it contributes only a single colony to the overall visible growth phenotype, but such a

cell in liquid -AD may give rise to a complete culture.

Further investigation of adaptation to -AD medium was felt to be required. The question of the proportion of cells of a population which contributes to the final growth curve was very difficult to resolve. It was hoped that, by plating on -AD in addition to YEA during a liquid growth experiment, the proportion of adapted cells in -AD liquid could be determined. If this were so, information concerning the number of cells giving rise to growth and also, perhaps, insight into processes occurring during the lag phase, might have been obtained. However, plating on -AD proved an ineffective method of reflecting events occurring in -AD liquid medium, probably because growth on -AD solid medium was determined by factors other than those governing growth in -AD liquid medium, i.e. ability to adapt to growth on -AD plates. Washed, stationary phase cultures from +ALL and -AD flasks (representing the situations before and after -AD growth, respectively) of Experiments 2 and 3 (see Fig. 3) were plated on -AD and YEA. -AD plates yielded slow-growing colonies of variable size, while colonies on YEA plates grew normally. Survival on -AD plates of -AD and +ALL cultures of Experiment 2 was approximately 100% that on YEA, while relative survivals of the 4 -AD cultures of Experiment 3 were 10%, 80%, 25% and 70%. Clearly, plating on -AD measures a parameter different from that required.

Streaking onto -AD of colonies from YEA plates of before and after -AD liquid growth to detect any proportionate increase in -AD plate growth ability was also tried without success (probably for the reasons given above in addition to the fact that growth on

non-selective medium prior to test-streaking was necessary).

Since questions about the size of the population producing -AD growth could not be adequately answered, further experiments concentrated on characterizing the adaptation response. The following questions were posed:

a) Is adaptation always maximal in -AD liquid medium, as suggested by the slope of the -AD growth curve?

b) Can $su_{D/+}$ diploids of very poor growth ability on -AD plates ever adapt to -AD liquid medium? If so, how long a lag is necessary?

c) Is growth on -AD solid and in -AD liquid medium always correlated? Are, for instance, the gradations of growth seen for various combinations of suppressor types reflected in gradations of lag period in -AD liquid medium?

By investigation such questions, it was hoped that a general picture of the system with which we were dealing could be formed.

iv) Investigations of the Adaptation Response

1. Experiments to determine whether the Response is always Maximal

Experiment 2 showed that on re-inoculation of a suppressed haploid into -AD, no lag was obtained, suggesting that adaptation during initial -AD growth was complete. However, the strain used in this experiment grew on -AD plates faster than did most suppressed haploids. Whether the lag could be totally abolished in strains initially of poor growth phenotype on -AD plates was unknown. Fig. 7b, representing growth curves of a strain previously selected on -AD plates, shows that a lag was present in -AD. There are several

possible explanations of this: in this strain, growth in -AD without a lag can never be obtained; selection on -AD plates was not complete; selection on -AD plates can never adapt cells completely to -AD liquid medium.

The following experiments were carried out to throw light on these possibilities. The deselected line of Experiment 3 was used for Liquid Growth Experiment 5: -AD1 and +ALLa (see Fig. 7a) cultures were inoculated into one fresh flask each of -AD and +ALL. Resulting growth curves are plotted in Figs. 9a,b. Plates corresponding to Fig. 9a could only be counted after 3 days, while those corresponding to Fig. 9b could be scored after 2 days.

Fig. 9a reveals that re-inoculation of a -AD culture into fresh -AD medium did not result in the elimination of the lag period, which was equal to, or perhaps even longer than, that in the reselected -AD flask. (The displacement of both the -AD and the corresponding +ALL curves to the right, due to low viability in the inocula, makes interpretation of the exact lag period in -AD difficult). Fig. 9b shows that the lag period in the -AD flask inoculated with unadapted cells is approximately 300 hours, longer than that found in Experiment 3 for the deselected line, but consistent with the assumption of variable adaptation times.

This long lag period prompted investigations to rule out the possibilities that growth was due either to a true ad_2 revertant or to contaminant yeast. Colonies from YEA plates of the stationary phase culture were therefore crossed to an $arg_{4.19}$ α strain and also streak-tested on -AD plates. The latter streaks grew poorly, an

Fig. 9a Liquid Growth Experiment 5

● +ALL

○ -AD

Inocula derived from -AD (1) culture of Liquid Growth Experiment 3 (see Fig. 7a)

Fig. 9a

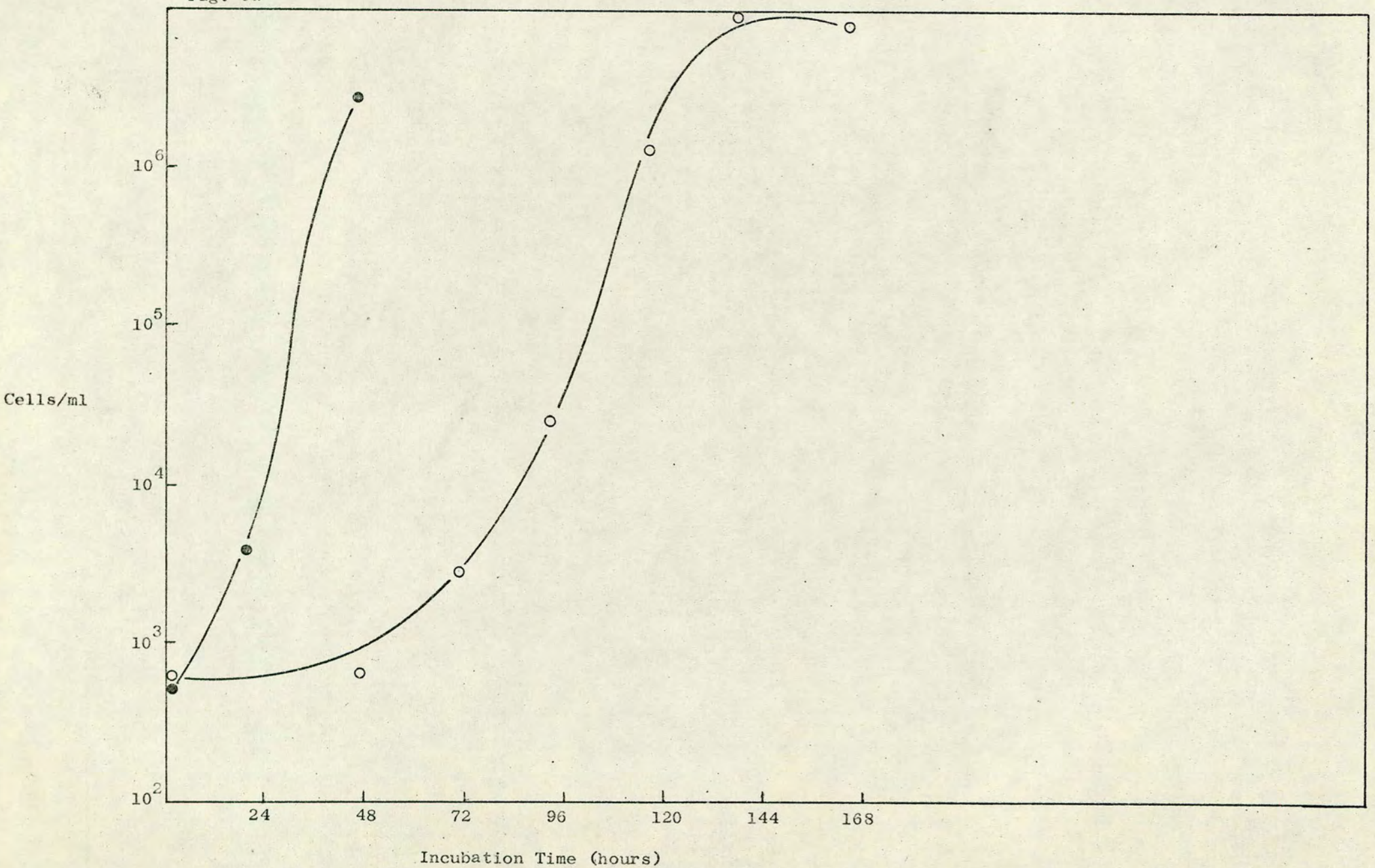

Cells/ml
10^6
10^5
10^4
10^3
10^2
24
48
72
96
120
144
168
Incubation Time (hours)

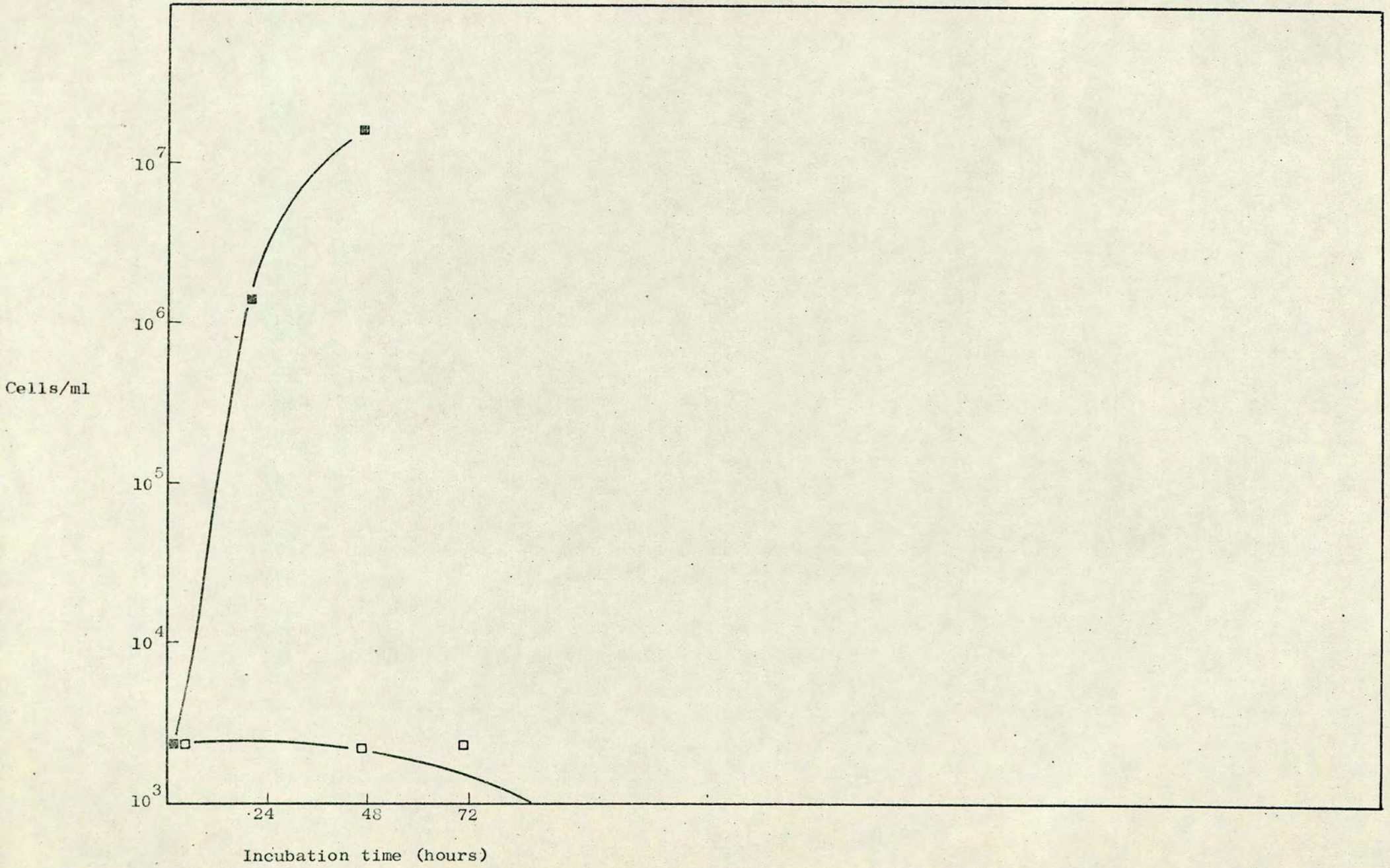
Fig. 9b **Liquid Growth Experiment 5**

■ +ALL

□ -AD (Growth first noted after incubation for 306 hours. Culture had reached late log phase by the following day but could not be plated out).

Inocula derived from +ALL 'a' culture of Liquid Growth Experiment 3 (see Fig. 7a).

Fig. 9b



indication that a true ad_2 revertant was absent. Sporulation of the above diploid, followed by plating of random ascospores, led to recovery of numerous pink, ad^- lys^- haploids. This eliminated any possibility that an ad_2^+ or a contaminant cell was responsible for growth after 300 hours incubation.

Thus it appeared that for this strain maximal adaptation was not easily attained, either by selection on -AD plates or by growth in -AD liquid medium, both of which reduced the lag, on subsequent -AD growth, to 0-48 hours. A possible explanation for the inability of this strain to grow without a lag in -AD might be found in terms of its genotype (i.e. suppressor or background genetic effects).

2. Liquid Growth Experiment 6: Lack of -AD Adaptation in $su_{D/+}$ Diploids

Considering the much poorer growth ability on -AD plates of $su_{D/+}$ diploids compared with su_H haploids, doubt existed whether adaptation in -AD liquid medium would be possible for the former, or whether the length of time required for the process would be greater than that for which non-dividing cells could survive in this medium.

Two independent suppressed 4c isolates were used in Liquid Growth Experiment 6. Isolate 'a' grew relatively well on -AD plates, independent colonies being distinguishable after 2 days; the few colonies growing on -AD streaks of isolate 'b' were only just visible after 4 days incubation.

5×10^5 cell/ml suspensions of the 2 isolates were made up using cells grown on +ALL plates. Each suspension was inoculated into one flask each of -AD and +ALL. Growth curves were plotted (see

Fig. 10).

Results for both 4c isolates were very similar. A rapid decline in viability in -AD was evident. -AD flasks were discarded after 356 hours when growth failed to occur.

The experiment was repeated using larger inoculations (up to 1.5×10^6 cells) of a suppressed 4c isolate. No growth was observed in any -AD flask after several weeks incubation.

3. Liquid Growth Experiment 7: Ability of $su_{D/+}$ Diploids to Adapt to -AD Liquid After Selection on -AD Plates

A suppressed $su_{D/+}$ isolate of 4c was selected for growth on -AD plates. Confluent growth of the selected -AD streak was achieved after 1-2 days. The unselected streak characteristically gave rise to only one or two individual colonies after 5 days. For Liquid Growth Experiment 7, 5×10^5 cell/ml suspensions of the above unselected and selected lines were made up and inoculated into one flask each of -AD and +ALL medium. Resulting growth curves are shown in Fig. 11.

Growth on YEA plates of the selected line was slower than that of the unselected line, requiring 4 rather than 3 days incubation before scoring. This lower growth rate was also apparent in +ALL liquid medium, as shown by the slopes of the exponential portions of the +ALL growth curves in Fig. 11. Both lines displayed a fairly rapid decline in viability upon inoculation into -AD. Whether or not such a decline continued could not be ascertained due to the impracticality of plating out as a means of determining viability of suspensions of less than 10^2 cells/ml with any degree of accuracy.

Fig. 10 Liquid Growth Experiment 6

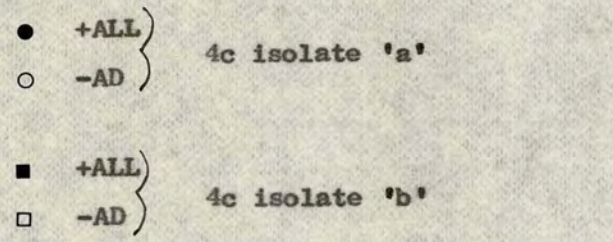


Fig. 10

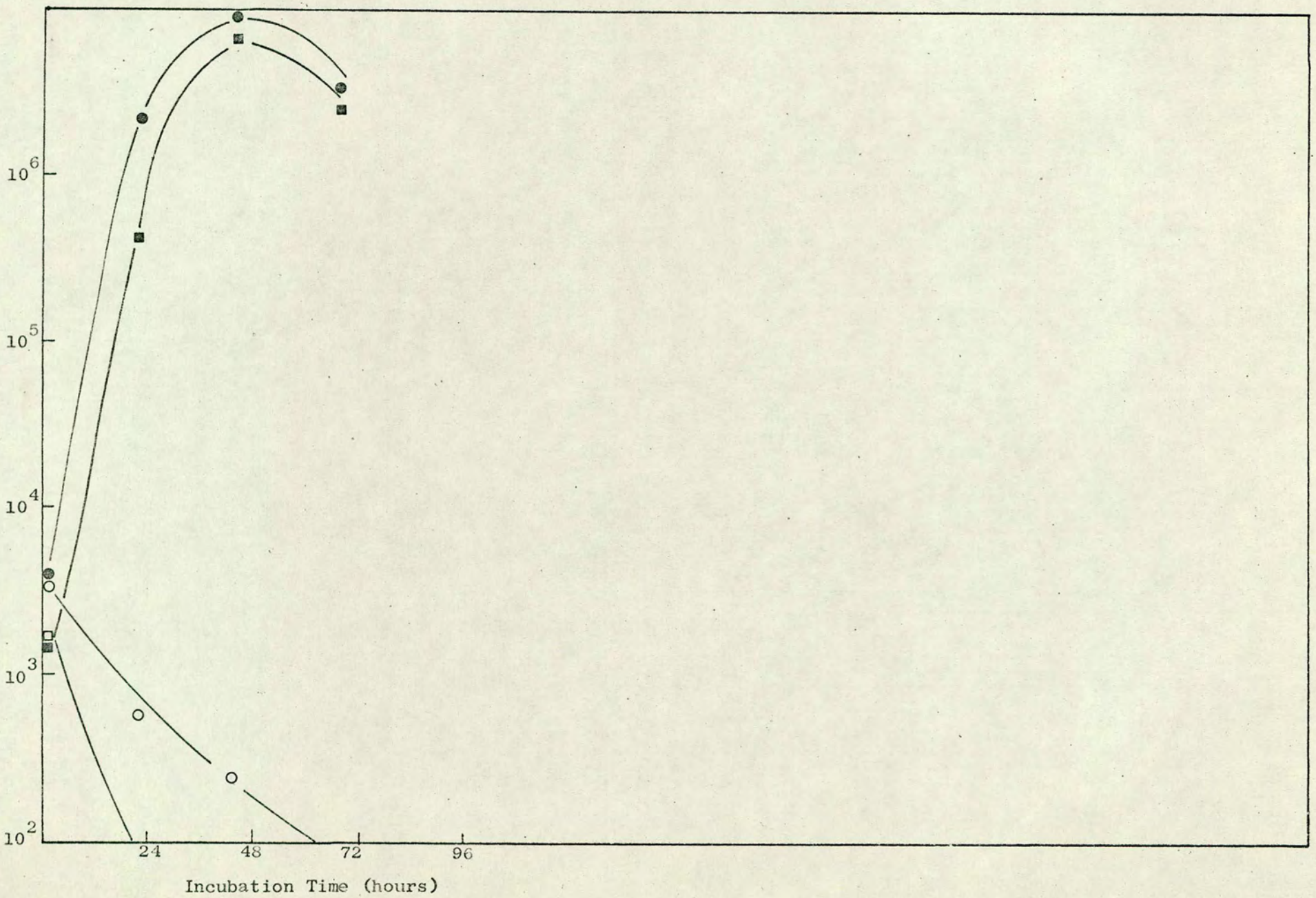
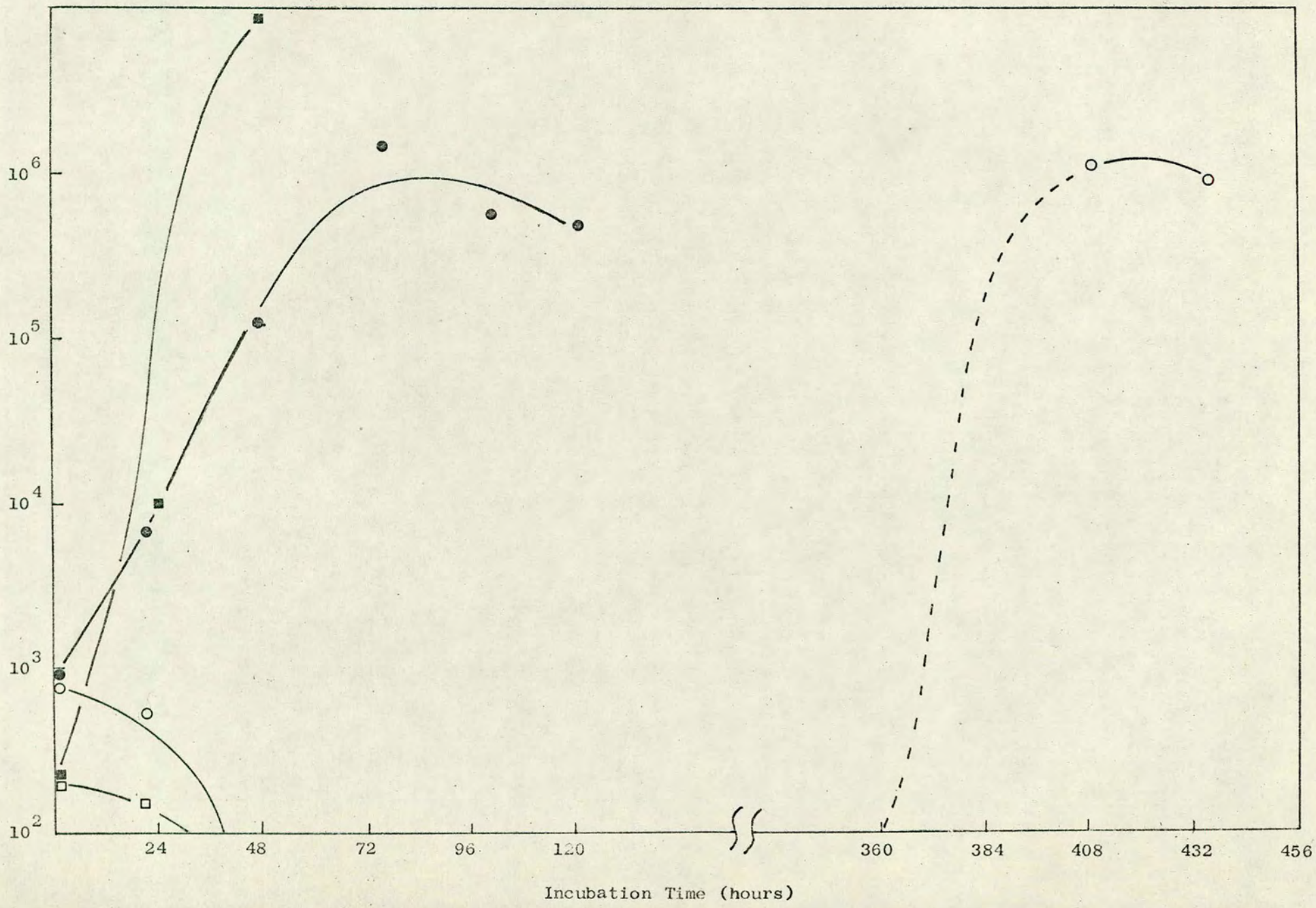


Fig. 11 Liquid Growth Experiment 7

■ +ALL
□ -AD
} Unselected 4c isolate

● +ALL
○ -AD
} Selected 4c isolate

Fig. 11


10^6
10^5
10^4
10^3
10^2
Cells/ml
24
48
72
96
120
360
384
408
432
456
Incubation Time (hours)

Growth of the selected line in -AD was observed after incubation for 400 hours. Unfortunately, the initial part of the growth curve was not determined. Microscopic examination of the cells responsible for growth revealed "giant" cells typical of 69/1-derived strains, thus ruling out contamination as the originator of growth. (Identification of the strain as leu^- met^-, considered too co-incidental for the genotype of a contaminant, supported this conclusion). It could only be concluded that growth was a consequence of either adaptation or true ad_2 reversion. Unfortunately, all attempts to sporulate the strain failed, so absence of a true ad_2 revertant could not be proven. However, this very lack of sporulation ability was interpreted as evidence in favour of an adaptation mechanism, in view of the reduced sporulation of selected diploids (see Section II c) ii) 1).

The surprisingly long lag period after which growth in -AD liquid was possible cast some doubt on results obtained in Experiment 6, in which -AD flasks were incubated for only 356 hours after inoculation. Since absence of growth in -AD in the latter experiment might have resulted from too short an incubation period, the -AD flask containing unselected cells in Experiment 7 was incubated for several weeks after growth in -AD of corresponding selected cells became apparent. However, despite this lengthy incubation, no growth occurred.

The experiment was repeated using selected and unselected lines of four additional $su_{D/+}$ isolates. While all four unselected lines failed to grow in -AD after approximately 8 weeks incubation, a range of responses was found for the corresponding selected lines. One isolate failed to grow at all, while the remaining three grew after

lag periods of 240 hours, 288 hours and 370 hours, respectively. This experiment was extended to confirm the results of Liquid Growth Experiment 2, in which pregrowth in -AD reduced the lag period on subsequent growth in -AD. Growth in -AD and +ALL flasks of two of the $su_{D/+}$ isolates was sub-inoculated into fresh -AD. No growth was obtained in -AD flasks inoculated with cells previously grown in +ALL. The -AD culture which grew originally after a 288 hour lag grew on subculture after a lag of only 48 hours, while the isolate which grew formerly after 240 hours displayed a 78 hour lag on subculture. A third -AD culture of the latter strain grew after a lag of 74 hours; the limit to which the lag could be reduced by prior growth in -AD appeared to have been reached. These experiments therefore supported the conclusion that growth in -AD liquid adapted strains to subsequent -AD growth.

Growth of $su_{D/+}$ diploids in -AD liquid medium thus appeared to require prior selection on -AD plates.

The contrast between results obtained in -AD liquid and on solid media is remarkable. Only the presence of agar distinguishes solid from liquid -AD medium. Nevertheless, solid -AD medium appears to be able to sustain growth of $su_{D/+}$ isolates far more readily than does liquid -AD. On solid -AD, growth of at least a portion of an unselected $su_{D/+}$ population has usually grown after 5 days (120 hours), while most cells of a selected population have probably grown within 48 hours. On the other hand, in liquid -AD, viability of $su_{D/+}$ isolates declines rapidly (see Figs. 10 and 11), behaviour not noted for suppressed haploid strains where death of the population

occurred more slowly. Unselected $su_{D/+}$ isolates appeared unable ever to grow in -AD liquid, while selected isolates grew in -AD liquid much later (after approximately 240-400 hours) than on -AD plates. No feasible explanation for these results can be suggested.

4. Liquid Growth Experiment 8: Correlation of -AD Lag with Genotype

It was previously reported (see Section I c) iii)) that $su_{H/+}$ diploids have a greater capacity for growth on -AD plates than do $su_{D/+}$ diploids, and $su_{H/+}$ $+/su_{D}$ diploids have a still greater ability. The behaviour in -AD liquid medium of $su_{H/+}$ and $su_{H/+}$ $+/su_{D}$ strains was thought worthy of investigation.

Selected and unselected isolates of several such strains were available. Typically, unselected $su_{H/+}$ isolates generated minute colonies on -AD streaks after about 4 days, while unselected $su_{H/+}$ $+/su_{D}$ isolates gave rise to intermittent growth on -AD after 3 days (see Plates 7a,b). All selected lines produced confluent growth on -AD after 2 days.

Selected and unselected lines of one $su_{H/+}$ and two different $su_{H/+}$ $+/su_{D}$ strains were used in Experiment 8. As usual, selected cells were taken from -AD and unselected cells from +ALL plates to make up suspensions for inoculation.

YEA plates used to monitor liquid growth of the $su_{H/+}$ isolate were difficult to score as colony size varied considerably. All plates were scored after 4 days incubation.

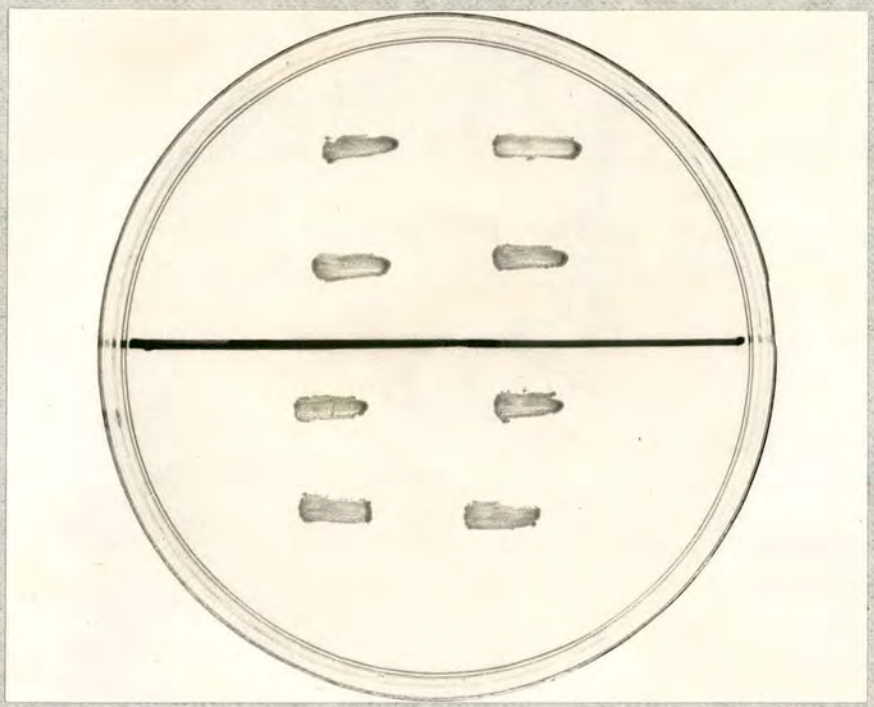
Plates of selected lines of both $su_{H/+}$ $+/su_{D}$ isolates were similarly difficult to score; colonies of isolate 'a' were counted

Plate 7 Growth Phenotypes of Unselected $\frac{su_H}{+}$ and $\frac{su_H}{+}\ \frac{+}{su_D}$ Isolates

Plates were incubated for 3 days

Photographs are approximately life-size

Plate 7



a. Growth on +ALL

4 streaks above line represent different $\frac{su_H}{+}\ \frac{+}{su_D}$ isolates

4 streaks below line represent different $\frac{su_H}{+}$ isolates

b. Growth on -AD

4 $\frac{su_H}{+}\ \frac{+}{su_D}$ isolates above line produce intermittant growth along streaks

4 $\frac{su_H}{+}$ isolates produce virtually no visible growth after 3 days incubation

after 5 days and those of isolate 'b' after 4 days. Plates of unselected lines were scored after 4 and 3 days respectively. Results are plotted in Figs. 12a,b.

Fig. 12a indicates that the selected $su_{H/+}$ strain grew in -AD after a lag of approximately 200 hours. The unselected control was incapable of such growth even after 8 weeks incubation.

Of the selected $su_{H/+}$ +/su_{D} strains, isolate 'a' grew after a lag of about 30 hours, while isolate 'b' grew with virtually no lag in -AD, as shown in Fig. 12b. Both -AD flasks inoculated with corresponding unselected isolates were incubated for 8 weeks, after which time no growth had occurred.

5. Summary and Discussion

Despite the limited number of diploid isolates tested, it was felt that the data, taken as a whole, allow certain tentative conclusions to be drawn.

Table 8 summarizes data on -AD lag periods obtained in various liquid growth experiments. The suppressed genotypes tested are arranged from left to right in order of increasing growth capacity of the unselected isolates on -AD plates. This order was obtained by subjective assessment of -AD growth capability of many different isolates with the above genotypes.

If it is accepted that the length of the -AD lag period is a valid reflection of the degree of adaptation to -AD growth ability, then it can be concluded from Table 8 that initial growth and the extent of adaptation possible for a particular isolate is limited by its genotype. Thus su_{H} haploids tend to grow better than any

Fig. 12a **Liquid Growth Experiment 8**

■ +ALL
□ -AD
} unselected $\frac{su_H}{+}$

● +ALL
○ -AD
} selected $\frac{su_H}{+}$

Fig. 12a

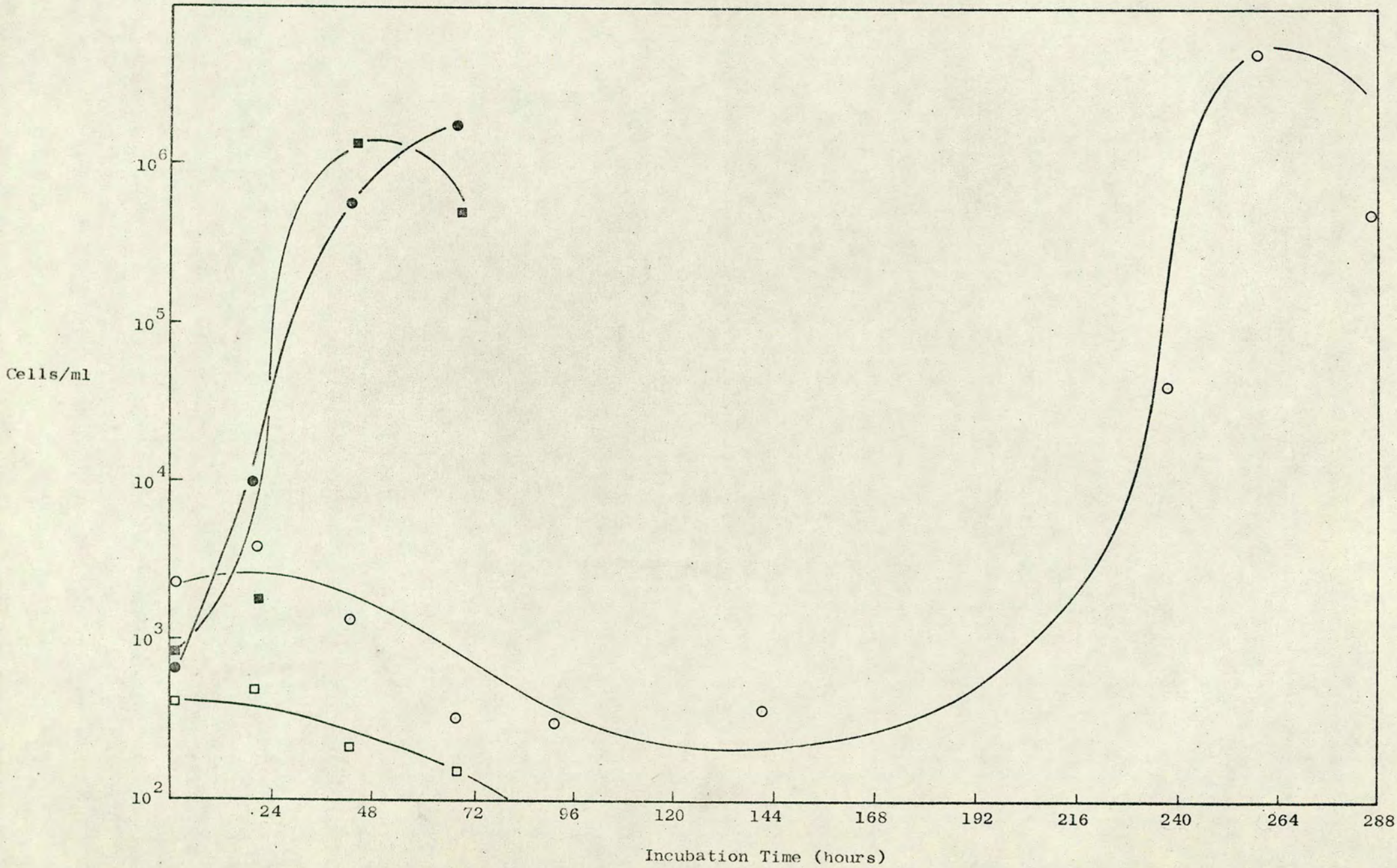

10^6
10^5
10^4
10^3
10^2
Cells/ml
24
48
72
96
120
144
168
192
216
240
264
288
Incubation Time (hours)

Fig. 12b **Liquid Growth Experiment 8**

● +ALL
▲ -AD
unselected $\frac{su_H}{+}\ \frac{+}{su_D}$ isolate 'a'

○ +ALL
△ -AD
selected $\frac{su_H}{+}\ \frac{+}{su_D}$ isolate 'a'

■ +ALL
✸ -AD
unselected $\frac{su_H}{+}\ \frac{+}{su_D}$ isolate 'b'

⊖ +ALL
✡ -AD
selected $\frac{su_H}{+}\ \frac{+}{su_D}$ isolate 'b'

Fig. 12b

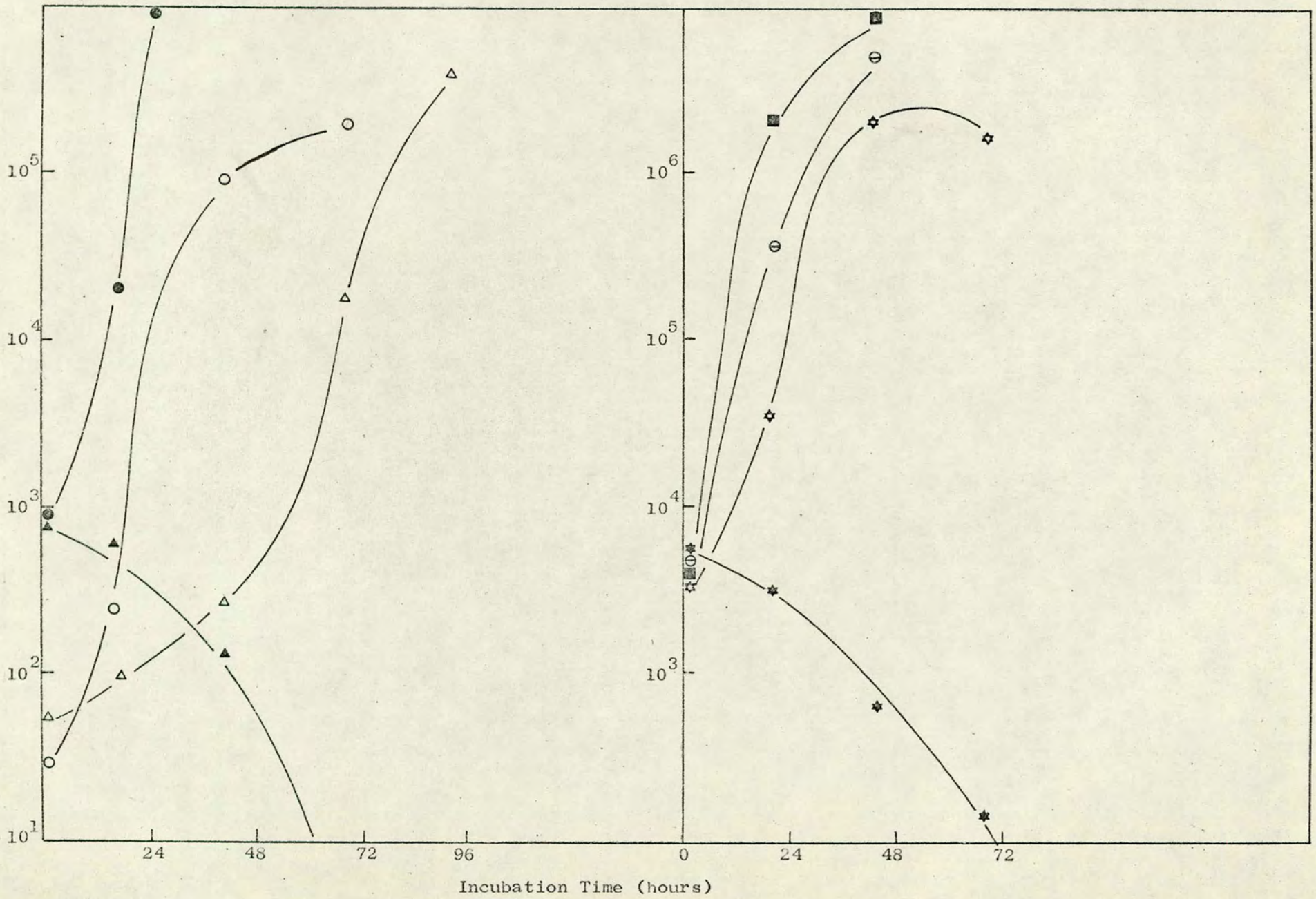

Cells/ml
10^1
10^2
10^3
10^4
10^5
10^6
24
48
72
96
0
24
48
72
Incubation Time (hours)

Table 8 Lag Periods in -AD found for Various Suppressed Genotypes

Table 8

Genotype / Treatment	$Su_{D/+}$	$Su_{H/+}$	$Su_{H/+}{}^{+}/Su_{D}$	Su_{H}
No selection on -AD plates	∞	∞	∞	24-300 hours
Selection on -AD plates	240-400 hours	200 hours	0-30 hours	0-48 hours

other suppressed genotype (except perhaps some $su_{H/+}$ +/su_D isolates when tested initially on -AD plates; only su_H strains can grow in -AD liquid medium without prior selection for growth on -AD plates. After such selection, the lag in -AD is either absent or of short duration. $Su_{H/+}$ +/su_D diploids grow to approximately the same extent as su_H haploids on -AD plates, but will grow in -AD liquid medium only when previously selected on -AD plates, showing no lag or one of about 30 hours. $su_{H/+}$ strains grow less well than do $su_{H/+}$ +/su_D diploids on -AD plates and also attain a lower degree of adaptation with selected, as shown by the lag of about 200 hours in -AD liquid. $Su_{D/+}$ strains generally grow least well of these genotypes on -AD plates; even after selection, the lag period in -AD liquid can be as long as 400 hours.

The range of lag periods found within each genotypic category is fairly large; however in most cases such ranges show no overlap between categories (an exception being those of su_H and $su_{H/+}$ +/su_D; these categories, however, are distinguished by the ability of only su_H strains to grow in -AD without prior selection on -AD plates).

To summarize, the genotype not only determines the unselected growth phenotype on -AD, but also imposes limits on the extent of modification of its own expression by non-genetic changes occurring during selection on, or growth in, -AD medium. Thus growth in -AD identical to that in +ALL liquid will only be achieved when the genotype is compatible with such behaviour. Moreover, although selection on -AD plates results in confluent growth within 1-2 days for all the suppressor genotypes considered above, the length of the

lag in -AD liquid still reflects the relative unselected growth capacity on -AD plates.

Section III: Nature of the Adaptive Response

The phenomena thus far described have been interpreted in terms of variations in cytoplasmic interactions occurring in the presence of a constant genetic background. It remained to elucidate the nature of the cytoplasmic changes involved in the selection and adaptation responses.

Two types of change could conceivably be involved: one that increases the concentration of enzyme specified by the AD-2 locus, phosphoribosyl-amino-imidazole carboxylase (PAIC) (Fisher, 1969) to levels allowing wild-type growth rate on -AD medium, or one that alters the specific activity of PAIC. (The possibility of induction of alternative enzyme pathways, by-passing the $ad_{2.1}$ block, was dismissed as improbable. The only pathway known to be capable of this is the histidine biosynthetic pathway; the flux from P-ribulosylformimino 5-NH_2-imidazole carboxamide-RP can supply 5-amino-4-imidazole-carboxamide ribotide (AICAR), a substrate formed after the $ad_{2.1}$ block, and thereby complete adenine biosynthesis (Queiroz, 1971). However, histidine inhibits the first enzyme in the histidine pathway by a feedback mechanism, AICAR production ceases, and the $ad_{2.1}$ block is not by-passed. Histidine was present in the -AD medium used, so such a by-pass probably does not exist in this system).

An increased concentration of PAIC might be a consequence of one of the following:-

1. Increased su^+ tRNA production, either specifically or via increased total tRNA synthesis, leading to increased suppression of

the $ad_{2.1}$ ochre mutant.

2. Increased proportion of a constant su^+ tRNA concentration reading the $ad_{2.1}$ ochre codon.

3. Increased suppression efficiency of su^+ tRNA due to, for example, greater activity of enzymes which modify su^+ tRNA bases, leading to improved ochre translation ability, or a change in cytoplasmic state favouring translation over termination at the nonsense codon.

Such changes in su^+ tRNA activity may have no detectable effect on suppression of other nonsense alleles if these are more "easily" suppressible than $ad_{2.1}$.

Similarly, an increase in the specific activity of PAIC might result from:-

4. Change in a component of the cytoplasmic milieu, e.g. pH, concentration of certain ions, which increases PAIC activity.

5. Charging of su^+ tRNA with a different amino-acid (caused by change in environmental conditions) with concomitant increase in PAIC activity.

It is difficult to reconcile the above possibilities with the apparent one-step nature of the adaptive response in -AD liquid medium and the cumulative effect of selection on -AD plates. However, a fuller consideration of these and other possibilities was felt to be inappropriate and, at this stage, lack of the necessary biochemical information made distinction between them impossible. Moreover, it must be pointed out that, valuable as it may be to identify the mechanism responsible for the effects described, the primary site of

action for selection or adaptation might still remain unknown.

Experiments involving quantitative and qualitative characterization of su^+ tRNA and PAIC were contemplated. Unfortunately, biochemical characterization of su^+ tRNA's was technically impractical in this laboratory, and no suitable assay for PAIC existed (Silver and Eaton, 1969). Other, indirect, methods had therefore to be used.

A preliminary question was posed concerning the practicality of selecting for suppressors relieving the effect of a block within the adenine biosynthetic pathway. It could be argued that, in the absence of endogenous as well as exogenous adenine available for tRNA synthesis, tRNA-mediated suppressor mutations arising in ad^- strains, potentially capable of suppressing the ad^- mutation, could not be immediately expressed on -AD medium. Without adenine, no su^+ tRNA can be synthesised, and without su^+ tRNA the adenine biosynthetic pathway cannot operate. This "vicious circle" may eventually be broken, and expression of the suppressor occur, following breakdown of cellular components containing adenine (e.g. DNA, RNA), or via a low level of UAA (or other nonsense codon) misreading. Once the mutant locus has been transcribed once, adenine biosynthesis begins, leading to the formation of su^+ tRNA molecules, which in turn cause increased transcription of the mutant ad^- gene and thus still more adenine production, thereby relieving the strain's requirement for adenine.

Such a situation could explain not only the low frequency of supersuppressors recovered on -AD plates, but might also account for the adaptive response in -AD liquid medium if the length of the

lag period represented approximately the time required for synthesis of initial su^+ tRNA molecules, which then increase sharply in number by positive feed-back, resulting very quickly in a wild-type growth rate.

There were several reasons the above hypothesis and its explanation of the -AD adaptive response were considered implausible, not the least of these being the supposition of the absence of an intracellular pool of adenine when cells are plated on -AD after growth in an adenine-rich medium. This fundamental assumption is almost certainly unwarranted. However, before rejecting the suggestion entirely, an experiment designed to test a prediction of the hypothesis was undertaken. The experiment, reported below, involved supplementation of -AD liquid medium with minute amounts of adenine. Such adenine would be expected to "prime" the suppression system allowing almost immediate growth of suppressed strains in -AD. A markedly reduced lag period in -AD in the presence of "primer" adenine would thus support the above hypothesis.

a) Liquid Growth Experiment 9: Effect of Low Level Adenine Supplementation

i) Determination of Appropriate Adenine Supplementation Level

An adenine supplementation level sufficient to "prime" the suppression system but insufficient to support significant growth in the absence of suppression was required. To this end, a missense (presumably unsuppressible) ad_2 haploid strain, $ad_{2.2}$ leu^-, was tested for growth ability in -AD liquid medium supplemented with 0.01, 0.1, 0.5 and 1 μg adenine/ml. Any growth occurring in such media in excess of that in -AD could be attributed to relief of

the adenine requirement per se (i.e. not to any mechanism indirectly leading to endogenous adenine production).

Two flasks each of -AD and +ALL, in addition to the above supplemented media, were used to obtain growth curves using the standard procedure.

Adenine concentration determined the growth rate and number of divisions which occurred before stationary phase was attained. At an adenine concentration of 0.01 μg/ml, approximately two divisions of the $ad_{2.2}$ leu^- population occurred before the onset of stationary phase. Growth curves obtained in -AD, +ALL and -AD + 0.01 μg adenine/ml are shown in Fig. 13. It was considered justifiable to assume that this latter supplementation level should be sufficient to "prime" the postulated system without allowing significant growth via mechanisms other than suppression.

ii) Effect of Adenine Supplementation

On the grounds that "priming" with exogenous adenine, if it occurs, would reduce the lag period in -AD of a suppressed strain, a haploid su^+ strain displaying an appreciable -AD lag was used. Any reduction in lag period of such a strain would be easily observed. For Liquid Growth Experiment 9, therefore, growth curves of this su^+ strain in -AD, -AD + 0.01 μg adenine/ml and +ALL were obtained. As an additional control, a su^- strain, otherwise isogenic with the su^+ strain, was similarly tested. This served as an internal control to show the extent of growth supported in such a strain by 0.01 μg adenine/ml supplementation in the absence of suppression. The results are shown in Fig. 14.

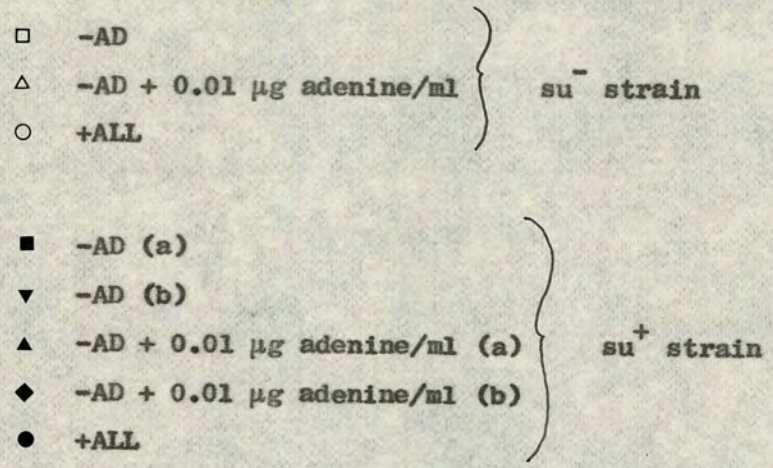
Fig. 13 Liquid Growth Experiment 9

○ -AD (2 replicates virtually identical)

△ -AD + 0.01 µg adenine/ml (ditto)

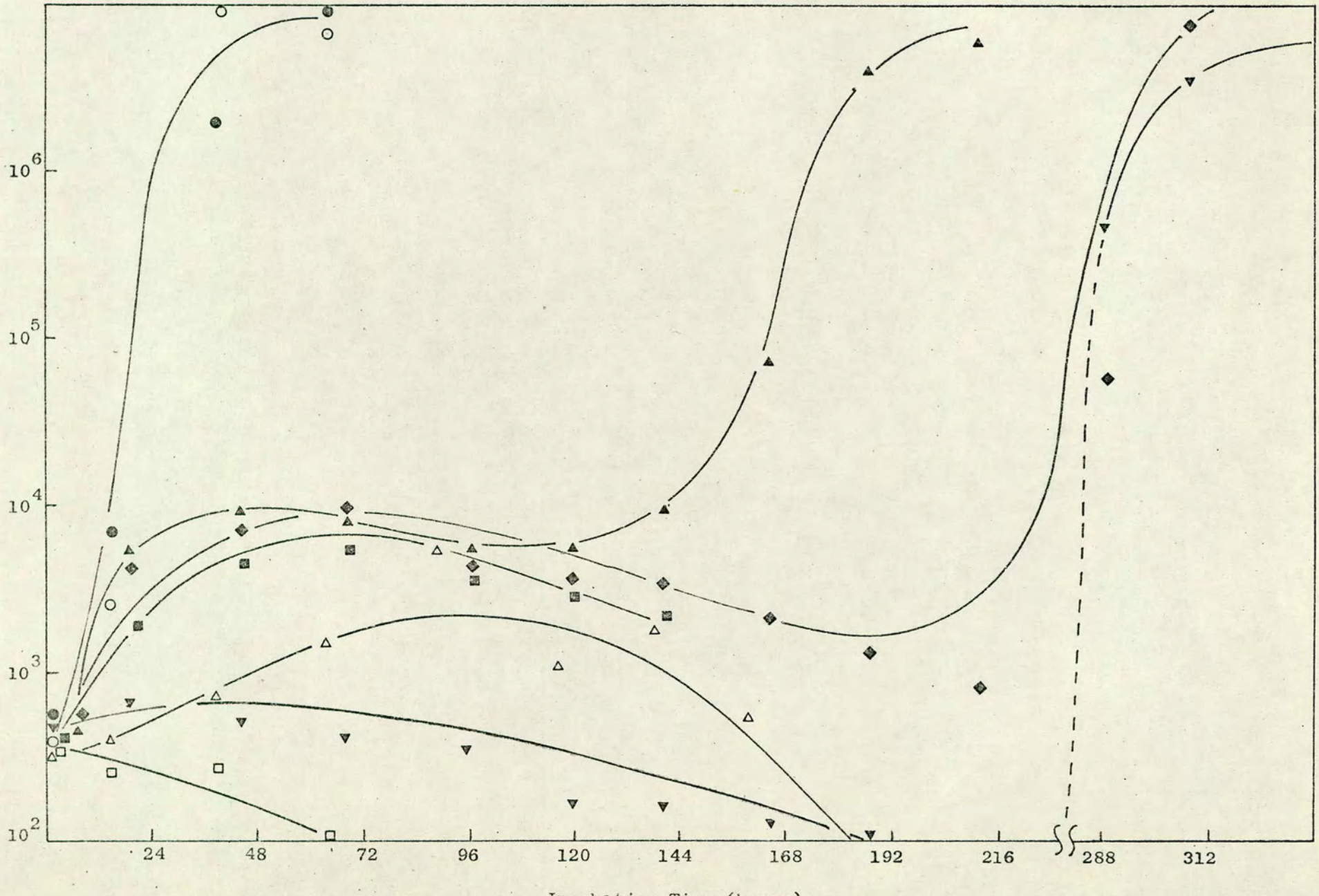
● +ALL (ditto)

Fig. 14 Liquid Growth Experiment 9



-AD (a) flask was found to be contaminated after 140 hours and was discarded

Fig. 14


10^6
10^5
10^4
10^3
10^2
Cells/ml
24
48
72
96
120
144
168
192
216
288
312
Incubation Time (hours)

0.01 µg adenine/ml supported 3 divisions of the su^- strain and 4-4.5 initial divisions of the su^+ strain. However, no significant effect of adenine supplementation on su^+ growth in excess of these early divisions was found: both relevant flasks supported growth after a long lag period, but a difference in lag of over 100 hours existed between the two flasks (the two lags being 132 hours and 287 hours, approximately). Su^- failed to grow in -AD, while one -AD flask inoculated with su^+ produced growth after 287 hours, a lag identical to that in one of the supplemented -AD flasks. The other -AD flask became contaminated before 140 hours incubation and was discarded. The above differences in lag period were consistent with a strain's variable adaptation time in -AD, previously proposed, rather than a requirement for adenine as "primer" for $ad_{2.1}$ suppression.

These results, therefore, suggest that requirement for intracellular adenine to give suppression is not a limiting factor in this system.

b) Adaptation Experiments with Strain 2885-32B

It will be recalled that an aim of this study was to identify the postulated cytoplasmic changes which occurred when suppressed strains were subjected to the physiological stress of exposure to -AD medium. The unfeasibility of direct measurement of su^+ tRNA and PAIC meant that indirect methods of investigation had to be employed. It was felt that examination of nonsense alleles, other than $ad_{2.1}$, which displayed a "leaky" suppressed phenotype, might provide a clue to the problem. No such mutants had been observed,

but strain 2885-32B, generously donated by Dr D.C. Hawthorne, which contained 4 as yet unstudied nonsense mutants, was available. Furthermore, one of these mutants, $ad_{5,7-101'}$, was the only ad^- nonsense allele, other than $ad_{2.1}$, which could be obtained. The behaviour of this allele was expected to throw light on the subject of section a) above. Furthermore, the existence of other suppressed nonsense alleles which can be manipulated in the same manner as $ad_{2.1}$ would point to the involvement of the suppression mechanism in such modification processes; if such a high frequency of enzymes whose activity could be modified by selection exists, then modification of penetrance for several of the many "leaky" missense alleles available in yeast should be well documented. This is not the case.

Investigations of suppressor activity in strain 2885-32B are reported below.

i) Recovery of Supersuppressors

Strain 2885-32B contains 4 ochre ($ad_{5,7-101'}$, $lys_{1.1}$, $ura_{4.1}$, $leu_{2.1}$) and 2 amber ($met_{8.1}$, $try_{1.1}$) alleles. In spontaneous reversion experiments, each of the 6 appropriate omission media yielded supersuppressors. Test-streaks of a random sample of revertants from each omission medium revealed the following:-

1) Suppression of $ad_{5,7-101'}$ was as efficient as that of $lys_{1.1}$ and $leu_{2.1}$, as judged by growth ability on the respective omission media.

2) Scoring of the phenotype depended to a great extent on the prior incubation period. For example, if scored after 3 days incubation, the vast majority ($^{45}/_{46}$) of lys^+, leu^+, ad^+ and ura^+

revertants appeared to contain an ochre-specific suppressor, while most ($^{13}/_{20}$) met^+ and try^+ revertants apparently harboured an amber-specific suppressor. However, after 4 or more days incubation, virtually all the latter revertants displayed a variable, though restricted, ability to suppress one or more of the ochre alleles. (This was inferred from the appearance on the streaks of a variable number of discrete colonies). Similarly, all 22 lys^+ revertants thought to contain an ochre-specific suppressor, showed very slight background growth or a variable number of single colonies on -MET and -TRY streaks after incubation for 5 or more days. Gilmore (1967) and Hawthorne (1969b) noted a similar dependence of phenotype on incubation period.

3) Growth on -URA was frequently observed to be less vigorous than that on -AD, -LYS and -LEU. This was the case whether the relevant suppressor was recovered as a suppressor of an ochre mutant or that of an amber mutant. In the latter case, where "leaky" growth of individual colonies was observed on -AD, -LYS and -LEU after 4 days, only slight background growth was seen on -URA streaks after 5 days.

10 su^- colonies were test-streaked on all omission media to determine whether the growth phenotypes described above were typical only of suppressed strains. A few colonies on -MET and -LEU streaks, most of which were due to suppressors, were obtained after a few days. No such growth on any other omission medium appeared. It was concluded that growth observed on streaks of suppressed strains was predominantly due to the presence of the initial suppressor gene.

As no mutator activity of suppressor genes in yeast was known, the late growth of streaks was tentatively attributed to inefficient suppression.

Observation 1) above implied that the phenomena reported for $ad_{2.1}$ suppression were not necessarily typical of all nonsense mutants in the adenine biosynthetic pathway. In addition, it reinforced the conclusion (reached in the first part of Section III) that "priming" with adenine was not a prerequisite for suppression of ad^- alleles since immediate and efficient expression of suppressors of $ad_{5,7-101}$ was apparently obtained in the absence of adenine supplementation.

Observations 2) and 3) were of greater interest for the extremely variable degree of suppression of the various nonsense alleles and the dependence of the phenotype scored on incubation time. Let us consider first observation 2). Most suppressors recovered were expected to be either ochre-specific or amber-specific, thus probably acting via altered tRNA species. The fact that most suppressors appeared to be of the amber-ochre variety, thought to involve components of the translation mechanism other than tRNA (Hawthorne and Leupold, 1974; Gerlach, 1975), was therefore unexpected. The following possibilities can be considered:-

1. The presence of the original suppressor increases the rate of mutation at other loci or is conducive to the phenotypic expression of other spontaneously occurring suppressors. The latter may be weak, by themselves being unable to produce suppressed growth.

2. Suppressors analogous to bacterial ochre suppressors (which effect translation of UAA and UAG by means of mutant tRNA) can be identified in yeast after prolonged incubation.

3. Omnipotent suppressors, not acting via tRNA, are almost exclusively obtained in this strain.

The latter two possibilities, of course, assume that late-growing colonies do not represent additional mutations, either true revertants or further suppressors, and that "slight background growth" does not result from phenomena unrelated to suppression. That the specificity of the suppressors involved in 2. and 3. above had apparently changed, on prolonged incubation, from either ochre-specific or amber-specific to amber-ochre specific, implied that one of the following situations may exist.-

i. Both amber mutants, $met_{8.1}$ and $try_{1.1}$, are very similar regarding the amino-acid substitutions they will tolerate at the site of the nonsense codon, as are the ochre mutants $ad_{5,7-101}$, $leu_{2.1}$, $lys_{1.1}$ and possibly also $ura_{4.1}$. However, these particular sets of amber and ochre mutants tolerate, in general, different amino-acid substitutions. Suppressors recovered by their action on an amber mutant insert the same amino-acid at UAG and UAA codons, but enzymes specified by amber nonsense loci will allow normal growth on relevant omission media, while those specified by ochre nonsense mutants have a low specific activity causing no growth or a leaky growth phenotype on appropriate omission media. The reverse situation, where suppressors of ochre mutants are recovered would similarly result in weak growth of amber mutants. The above

situation requires that amber-ochre suppressors insert specific amino-acids, and are therefore most unlikely to be "omnipotent" suppressors acting via a ribosomal protein component.

ii. Specificity of recognition of UAA and UAG codons may be involved. Suppressors may recognise and translate one codon far more frequently than the other. For instance, UAA may be translated at a high frequency, only a brief incubation period being necessary for expression of this ability; UAG, however, may only be recognised and translated inefficiently, prolonged incubation being required before expression of such weak suppression ability can occur. Such a situation could be envisaged for either ribosomal or tRNA-mediated suppression.

If either of possibilities 2. and 3. above were true, studies with suppressed isolates of 2885-32B were thought potentially valuable in assessing whether certain tentative conclusions regarding the behaviour of suppressed $ad_{2.1}$ strains were feasible. It was hoped that selection for increased growth ability of the "secondary" mutants (i.e. those of the nonsense type not used in the recovery of the suppressor, and showing "leaky suppression") might be possible. If it were, and other secondary mutants displayed a correlated response, and the selection of additional mutations could be ruled out, then the effects of selection could be ascribed to cytoplasmic modifications of the suppression system. Altered codon-recognition specificity (as for situation ii. above) or increased suppression efficiency leading, via increased concentrations of enzymes of low specific activity, to increased total enzyme activity (as for situation i. above)

are characteristics of the suppression system theoretically modifiable by a change in cytoplasmic state. Such a demonstration of cytoplasmic control over certain aspects of the suppression system would lend credibility to the interpretation of $ad_{2.1}$ suppression phenomena in terms of a similar mechanism. If, however, no correlated responses to selection could be found, but only increased growth ability at a single locus, adaptation of biochemical pathways would appear more probable.

That the "leaky" suppression of $ura_{4.1}$ (observation 3) above) could be analogous to that of $ad_{2.1}$ was considered possible. Selection for a progressive increase in growth capacity on -URA would (as previously suggested) lend support to the hypothesis that adaptation of the suppression system, rather than that of a biochemical pathway, can occur. On the other hand, as $ura_{4.1}$ is often as well suppressed as any other allele, it may be that the enzyme specified by the URA 4 locus is more stringent in its amino-acid requirement at the site of the nonsense codon than are the other ochre mutants used (a possibility consistent with the observation that ura^{+} revertants were recovered at a lower frequency than were those of $lys_{1.1}$, $leu_{2.1}$ and $ad_{5,7-101}$'). If this is the case, leaky growth on -URA could result from very low activity of the enzyme specified by suppressed $ura_{4.1}$ despite a possible high suppression efficiency.

Assuming success of the experiments with 2885-32B proposed above, an extension of such studies, involving crossing in of the $ad_{2.1}$ allele into 2885-32B, enabling detection of any correlated responses to selection between $ad_{2.1}$ and the other nonsense alleles, was envisaged.

With these considerations in mind, selection experiments were performed as detailed below:-

1. Selection for growth ability of a given suppressed 2885-32B isolate was carried out by restreaking from leaky growth (and, as a control, from growth on +ALL) onto all omission media. Acquisition of good growth via a single restreak on the selective medium was thought likely to be due to the selection of additional revertants, and such isolates were discarded. If correlated changes of phenotype occurred during selection for a single growth phenotype, the isolate was analysed further as described below.

2. Relaxation of selection pressure by growth of selected lines on YEA, followed by test-streaking on each omission medium, determined whether the processes involved in the selection response were easily reversible. Whereas a reversal of the selection effect upon relaxation of selection pressure would indicate cytoplasmic involvement in the effect, vegetative stability of the selective effect under these conditions was not unambiguous evidence for the involvement of genetic changes in the effect. Therefore, where no "deselection" occurred upon relaxation of selection pressure, it was necessary to analyse the genetic situation further by studying meiotic segregation patterns.

3. Both selected and unselected lines were outcrossed in an attempt to determine whether or not the effects of selection survived meiosis. While accepting the possibility that non-genetic cytoplasmic changes might be sufficiently stable to withstand the molecular rearrangements involved in diploidization and even meiosis,

so that survival of the selection effect into the diploid could not be taken as unequivocal evidence for the selection of a suppressor gene, it was considered that disappearance of the effect in the diploid and its progeny could be fairly safely explained on the basis of a cytoplasmic involvement in the selection effect. Analysis of the haploid progeny of the diploid formed was expected to indicate the number of suppressors present, and hence provide further information on the cause of the selective effect. The procedure was as follows. A strain isogenic but for mating-type to 2885-32B was not instantly available. While it was being constructed, a $lys_{1.1}$ try^{+} or a $lys_{1.1}$ met^{+} strain was utilized. (In either case, the rationale used in the determination of number of suppressors present was the same). In the former case, the diploids synthesized had the genotype $\frac{lys_{1.1}}{lys_{1.1}} \; \frac{try_{1.1}}{+}$. Unfortunately, as only $lys_{1.1}$ was homozygous, the diploids formed could not be tested for dominance of the selected activity. Analysis of the situation was therefore confined to a study of the spore progeny.

Let us consider the case where a strain apparently carrying an ochre-specific suppressor has gained, via selection, amber-suppressing ability. There are several possible causes for this acquisition:

1) An amber-specific or amber-ochre suppressor has been picked up.

2) A modifier mutation at, or external to, the suppressor locus bestowing amber-reading ability on the suppressor is present.

3) Dominant or recessive cytoplasmic changes conferring amber-reading ability have occurred.

(Complications may arise when, during the course of selection,

still more suppressors are selected; for the sake of simplicity we assume these cases to be rare and the possibility can be ignored).

It was thought that these various possibilities might be distinguishable by means of tetrad analysis. Table 9 illustrates the use of segregation patterns of lys^- and try^- in distinguishing between the above possibilities. Tetrad analysis of the control (unselected) line crossed to, for instance, $lys_{1.1}$ try^+, should give a $2^+:2^-$ ratio at both loci, and lys^+ try^- spore clones should be recovered. In the case of the selected line crossed to $lys_{1.1}$ try^+, we have different expectations according to the nature of the amber-suppressing ability. If only one suppressor, su_{a-o}, modified by mutation at the suppressor locus to suppress both amber and ochre alleles is present, whenever $lys_{1.1}$ is suppressed, so will be $try_{1.1}$; no lys^+ try^- spores will be recovered. If 2 suppressors are present, the original ochre-specific suppressor, su_o, and an additional suppressor capable of suppressing amber alleles, su_a (amber-specific) or su_{a-o} (amber-ochre specific), spores should arise which contain su_o and $try_{1.1}$ but not su_a (or su_{a-o}). Such spores will be lys^+ try^-. The presence of such haploids, in which ochre and amber suppression ability have been separated, therefore distinguishes $su_o + su_a$ (or $su_o + su_{a-o}$) from su_{a-o} alone in the selected lines. (If the additional suppressor is su_{a-o}, more than 2 lys^+ spores per tetrad should occur).

In a similar way, expected segregations in the presence of an external modifier mutation, or recessive or dominant cytoplasmic states causing modification of the suppressor's activity, can be deduced. Considering again the case where an ochre-suppressed

Table 9 Possible Segregations in Diploids of Selected Suppressed 2385-32B x lys^- try^+

Diploid Genotype : $\frac{lys_{1.1}}{lys_{1.1}}$ $\frac{try_{1.1}}{+}$

✓ : presence of lys^+ try^- spores

\- : absence of lys^+ try^- spores

Table 9

Cause of Selective Effect		Direction of Selected Suppressing Activity					
		Ochre→ Amber			Amber→ Ochre		
		lys^+:lys^-	try^+:try^-	Presence of lys^+ try^-	lys^+:lys^-	try^+:try^-	Presence of lys^+ try^-
Additional	Amber-specific or ochre-specific	2:2	2:2 3:1 4:0	✓	2:2	2:2 3:1 4:0	✓
Suppressor	Amber-ochre specific	2:2 3:1 4:0	2:2 3:1 4:0	✓	2:2	2:2 3:1 4:0	-
Internal Modifier		2:2	2:2 3:1 4:0	-	2:2	2:2 3:1 4:0	-
External Modifier		2:2	2:2 3:1 4:0	✓	2:2 1:3 0:4	2:2 3:1 4:0	-
Cytoplasm	recessive	2:2	2:2	✓	0:4	2:2 3:1 4:0	-
	dominant	2:2	2:2 3:1 4:0	-	2:2	2:2 3:1 4:0	-

strain acquires amber-suppressing ability, the selected line will yield lys^+ try^- progeny if a recessive cytoplasmic state or an external modifier gene caused the selection effect. If dominant cytoplasmic conditions were the cause, no such spores should be recovered.

In the situation where an amber-suppressed strain gains ochre-suppressing ability, lys^+ try^- spores are not expected in any of the above 3 cases. If a new ochre-specific suppressor is responsible for the selection effect, it will be identified as described above. However, if a new su_{a-o} is responsible, no lys^+ try^- spores should arise (and lys^- try^+ spores do not necessarily indicate the presence of only the original su_a). In this case, therefore, su_a + su_{a-o} cannot be distinguished from a single su_{a-o}. This applies also to selection for a wider suppression spectrum of su_{a-o} (in which a selected increase in suppression efficiency might lead to correlated increases in ability to suppress several different nonsense alleles), where an additional su_{a-o} is selected. However, as a first step in analysing the situation, this method of determining the number of suppressors present was considered adequate.

Despite the diversity of situations theoretically able to give rise to a progressive improvement of suppression during selection, it was felt that, bearing in mind the frequency (about 70% of the suppressed isolates tested) with which a slow or leaky growth phenotype was observed, the 2 most likely causes would be recessive cytoplasmic adaptation and the aquisition of a second suppressor gene. These should be easy to distinguish using the above procedure. If required, a more detailed study could be carried out when more

appropriate strains become available.

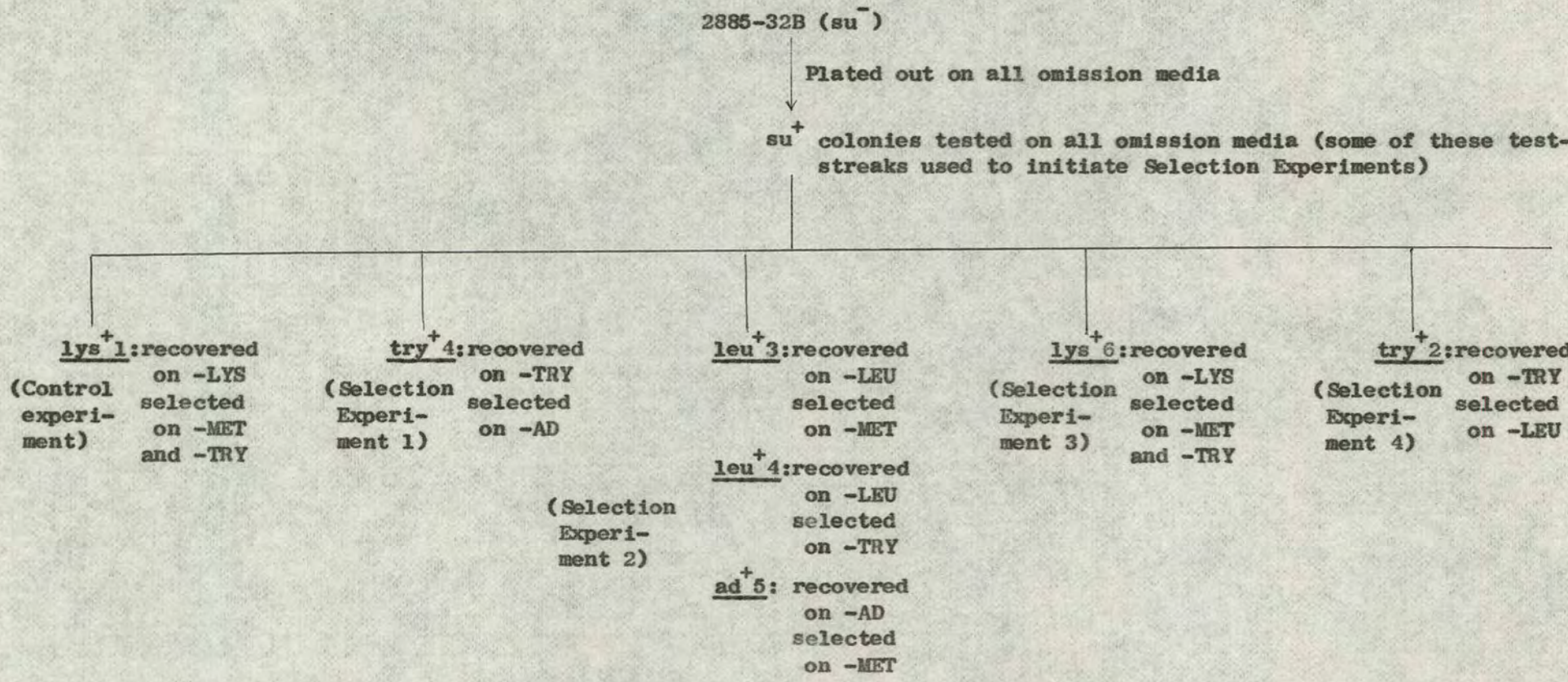
6 selection experiments, involving 12 different suppressed isolates, were carried out; 2 attempted to convert an amber-suppressing capacity into an amber-ochre-suppressing one, 2 to convert ochre into amber-ochre suppressing capacities and 2 to extend by selection the suppression range (i.e. the number of nonsense alleles suppressed) of an existing amber-ochre suppressor. Of the 6 experiments, one failed to show a selection effect, one showed aquisition of the desired phenotype after one restreak and probably contained a second suppressor or a modifier mutant, 3 showed a gradual aquisition of ability to suppress the appropriate alleles (although far more rapid than that found in $ad_{2.1}$ selection experiments), and one gave results probably best accounted for in terms of a single base change in the anticodon of the su^+ tRNA, leading to its recognition of UAA rather than UAG. The latter 4 experiments, in all of which correlated changes to selection were found, are described more fully below. The relationships between isolates used in these selection experiments are shown in Fig. 15.

The general impression was gained that the presence of an initial suppressor increased the probability of recovering a second one, but this aspect was not investigated.

A preliminary set of experiments was carried out to determine whether a strain carrying 2 suppressors did indeed display the characteristics assumed above.

Isolate "lys^+1" was recovered from -LYS. Its phenotype was scored after incubation for 2 days as ad^+ lys^+ leu^+ ura^+. A few

Fig. 15 **Relationship of Suppressed 2885-32B Isolates Used in Selection Experiments**



small colonies on -MET and -TRY streaks became apparent the following day. Restreaking of such colonies led immediately to confluent growth on all omission media. The presence of 2 suppressors was suspected. Deselection on YEA for over a month failed to restore the original phenotype on -MET and -TRY. Unselected lys^+1 and an isolate selected from -MET were grown in liquid omission media. Both unselected and selected isolates grew normally (with no lag) in -AD, -LYS, -LEU and +ALL. In -MET, the unselected isolate grew only after a considerable lag (270 hours), while the selected isolate grew with no lag in this medium. The significance of the lag period in this instance is not clear. Such behaviour, however, was thought consistent with the presence of 2 suppressors, capable of suppressing ochre and amber alleles respectively, as the activity of two such suppressors might be expected to be additive.

Selected and unselected lys^+1 isolates were crossed to a lys^- try^+ strain. 2 out of the 19 spores isolated from the cross to unselected lys^+1 were lys^+ try^-. 6 tetrads from the cross to selected lys^+1 gave $2^+:2^-$ segregation at the lys^- locus and $3^+:1^-$ and $4^+:0^-$ at the try^- locus; 2 lys^+ try^- spores were found. Such segregations were those expected for a $\frac{lys}{lys}\ \frac{try}{+}$ diploid in the presence of an ochre-specific and an amber-specific suppressor (or, less likely, an su_o and an external modifier gene enabling the suppressor to suppress amber alleles as well).

The behaviour predicted for a strain thought to be carrying both ochre and amber suppressors was thus confirmed.

Selection Experiment 1

When first recovered, the isolate "try$^+$4" was classified as ad$^-$ lys$^-$ leu$^+$ ura$^-$ try$^+$ met$^+$; an amber-ochre suppressor appeared to be present. Incubation for over 5 days resulted in slight background growth of the -AD streak. Subsequent selection from this growth led, in about 2 steps, to a confluent growth phenotype on -AD after incubation for one day and a less vigorous growth phenotype on -LYS after incubation for 2 days. (It was noted that the selected line always grew more slowly on +ALL than did the control line). The presence either of 2 suppressors or of a single suppressor with an extended range of suppression (due to modification by a modifier gene or cytoplasmic conditions) was thought possible. Outcrossing of the unselected line to lys$_{1.1}$ try$^+$, followed by analysis of 9 tetrads, produced no lys$^+$ spores. On analysis of 9 tetrads from the diploid made with the selected line, 2$^+$:2$^-$, 3$^+$:1$^-$ and 4$^+$:0$^-$ segregations of try$^-$ were found. At least 2 lys$^+$ try$^-$ spores were recovered. Only the presence of an additional ochre-specific suppressor could adequately explain these results and investigation of this isolate was discontinued.

Selection Experiment 2

"Leu$^+$3", "leu$^+$4" and ad$^+$5" isolates grew on -AD, -LYS, -LEU and -URA, but not on -MET or -TRY; they were classified as containing ochre-specific suppressors. However, after approximately 10 days incubation, discrete colonies were found on the -MET streaks of leu$^+$3 and ad$^+$5 and on the -TRY streak of leu$^+$4. Selection from these colonies was carried out. After only 1-2 restreaks, confluent growth

on the relevant medium was attained after incubation for one day; growth on the remaining omission medium, for which there had been no direct selection, was less good but present after incubation for 2-3 days.

Selection pressure was relaxed by restreaking selected lines on YEA every day for 18 days, test-streaking on relevant media every 5 days. Both unselected and selected lines were continued for comparison. No effect of relaxation of selection pressure could be demonstrated for leu^+3 or ad^+5, but leu^+4 did appear to lose a certain degree of suppression ability, in that the selected line grew on -TRY after incubation for one day, while the deselected (relaxed) line grew poorly on -TRY after 3-4 days incubation.

Of approximately 100 random spores from the cross between unselected leu^+4 and $lys_{1.1}$ try^+ $arg_{4.17}$, only 5 were of the lys^+ try^- phenotype. This result was unexpected; the minimum number of lys^+ try^- are expected when su_o and try^- are unlinked, when 25% spores should have this phenotype. The low number of lys^+ try^- spores is unexplained; possibly, in this cross, try^- spores have a low viability.

A selected line of leu^+4 was similarly crossed. 30 viable spores, comprising 4 tetrads, 4 triplets and one doublet were recovered from the 9 asci dissected. All spore-clones were test-streaked. Results are shown in Table 10. Segregation of the two heterozygous, ochre-suppressible alleles, $arg_{4.17}$ and $ura_{4.1}$, was that expected in the presence of a heterozygous su_o. Segregation at both the TRY 1 and LYS 1 loci was $2^+:2^-$ in all tetrads; no more

Table 10 Segregation in Selected $leu^{+}4 \times lys_{1.1}$ try^{+} Diploids

Diploid Genotype : $\frac{+}{arg_{4.17}}$ $\frac{lys_{1.1}}{lys_{1.1}}$ $\frac{ura_{4.1}}{+}$ $\frac{try_{1.1}}{+}$

\+ : growth

\- : no growth

Table 10

Tetrad, triplet or doublet		-ARG	-LYS	-URA	-TRY	+ALL
1	a	+	-	+	+	+
	b	+	+	+	+	+
	c	+	+	+	-	+
	d	+	-	-	-	+
2	a	+	+	+	+	+
	b	-	-	-	-	+
	c	+	+	+	+	+
	d	-	-	-	-	+
3	a	+	+	+	+	+
	b	+	-	+	+	+
	c	-	-	-	-	+
	d	+	+	+	-	+
4	a	-	-	-	+	+
	b	-	-	-	-	+
	c	+	+	+	+	+
	d	+	+	+	-	+
1	a	+	-	+	+	+
	b	+	+	+	+	+
	c	+	+	+	-	+
2	a	+	-	-	+	+
	b	-	-	+	-	+
	c	+	+	+	+	+
3	a	+	+	+	+	+
	b	-	-	-	+	+
	c	+	+	+	-	+
4	a	+	-	+	+	+
	b	-	-	+	-	+
	c	+	+	+	+	+
1	a	+	+	+	+	+
	b	+	-	-	-	+

than 2 try^+ or lys^+ spores were present in any of the triplets. Segregation patterns at the 2 loci were not co-incidental, however, and 5 lys^+ try^- spores were present. Since these lys^+ try^- spores were found, dominant cytoplasmic modifications, or a modifier mutation at the suppressor locus, could be rejected as the cause of the selection effect.

$Try_{1.1}$ is centromere-linked. A postulated su_a or su_{a-o}, if linked to $try_{1.1}$, would give $4^+:0^-$ segregation for tryptophan requirement. This clearly is not the case (see Table 10). If such a suppressor were unlinked to both $try_{1.1}$ and its own centromere, the expected ascus types are in the proportions $^1/_6$ non-parental ditype (NPD), $^1/_6$ parental ditype (PD) and $^2/_3$ tetratype (TT). Thus the probability of obtaining the 4 NPD asci found is $(^1/_6)^4$ or $^1/_{1296}$, any linkage between $try_{1.1}$ and the suppressor decreasing this probability still further. The remaining possibility, that the suppressor is centromere-linked on another chromosome, must also be considered. Assuming, as the simplest case, complete linkage of both $try_{1.1}$ and the suppressor to their respective centromeres, only PDs and NPDs will be obtained, and these with equal frequency. PDs are recognized by a $4^+:0^-$ segregation for tryptophan-requirement. Table 10 illustrates that no such ascus type was obtained amongst the 9 asci dissected. The probability of this occurrence is $(^1/_2)^9$.

That only NPD asci produce four viable spores, thereby causing a distortion in ascus types obtained, is improbable for two reasons: firstly, the spore inviability found is not sufficiently high and secondly, there is no evidence for the inviability of any try^+ phenotype.

Despite the low number of asci scored, the low probabilities of obtaining the observed results if a su_a or su_{a-o} were present resulted in the rejection of such a possibility.

Thus either a modifier external to the suppressor locus or recessive cytoplasmic modifications were concluded to have been responsible for the effect of selection. The limited number of spores tested meant that these 2 possibilities could not be definitely distinguished. However, the facts that an external modifier could have given rise to more than 2 try^+ clones per tetrad, but no such cases were found, and that maximum amber-suppressing ability was not accomplished in one step, while relaxation of selection pressure caused an apparent reduction in suppression efficiency, pointed to the involvement of cytoplasmic adaptations, which disappear during diploidization or meiosis. If so, the premise that adaptation in suppressed $ad_{2.1}$ strains could be due to adaptation of the suppression system, is supported.

Selection Experiment 3

Isolate "lys^+6" was scored, after incubation for 4 days, as ad^+ lys^+ leu^+ ura^+ met^- try^-. An ochre-specific suppressor appeared to be present. After approximately 7 days, slight background growth on -MET and -TRY streaks became apparent, and after 10 days small colonies growing along the streaks could be discerned. This growth, from both -MET and -TRY streaks, was selected. On the first restreak, no consistent effect of selection was seen. Selection was continued, using slow growth on -MET streaks. Slight growth on -MET after 2 days incubation was obtained on the second restreak. After 8 further

restreaks, lys^{+}6 grew well on -MET after incubation for 2 days.

When tested on the remaining omission media, interesting results were obtained: growth of the selected line on -AD, -LYS, -LEU and -URA was poor compared with that of the unselected line when scored after incubation for one day (this difference being far less obvious after 2 days).

It was noticed that the selected lys^{+}6 line now displayed a "leaky" growth phenotype on -TRY not shown by the unselected line. After 6 more restreaks from -MET, the selected line of lys^{+}6 grew on -MET and -TRY after incubation for only one day. (Incubation time necessary for -TRY growth subsequently varied from 1-3 days). It is proposed that this provides clear evidence for correlated selection for growth ability not easily explicable merely in terms of accumulation of additional suppressor genes.

After 5 further restreaks, ability of the selected line to grow on -MET and -TRY was unchanged, but that on -AD, -LYS, -LEU and -URA was further reduced, the differences in growth between selected and unselected lines being observable even after incubation for several days.

Both relaxation of selection and reselection for ochre-suppressing ability, however, failed to give the original phenotype of strong ochre and very weak amber suppression.

In an attempt to quantify the changes which had occurred in the selected line, both selected and unselected lines of lys^{+}6 were grown in all the relevant liquid omission media, and growth curves were constructed. Suspensions were made up from -LYS (for the

unselected line) and -MET (for the selected line). Each suspension was inoculated into one flask each of -AD, -LYS, -LEU, -URA, -MET, -TRY and +ALL. Growth curves for Liquid Growth Experiment 10, shown in Fig. 16a,b, were obtained using the standard technique.

Since, in 2885-32B, many suppressors were recovered on all omission media compared with the number on -AD for 69/1-derived strains containing $ad_{2.1}$, the possibility of growth due to additional suppressors must be borne in mind when interpreting these growth curves. However, assuming, as for 69/1-derived strains, that the length of the lag period reflects some aspect of ability to grow on a given medium, the results in Fig. 16a,b support those found on solid media. Fig. 16a, representing unselected lys^+6, shows relatively good growth in -AD, -LYS and -LEU, and poor growth in -MET, -TRY and -URA. In comparison, in Fig. 16b, representing selected lys^+6, only growth in +ALL and -LEU remains the same; growth ability in -MET and -TRY is improved, while that in -LYS and -AD is reduced and that in -URA absent altogether.

It is the relative positions, rather than the actual lag periods, of the curves that are interesting: in Fig. 16a, -MET and -TRY curves are to the right of those of -AD, -LYS and -LEU, but in Fig. 16b, the -MET curve is very similar to that of -LEU and to the left of -AD and -LYS, while the -TRY curve is to the left of -AD. In other words, all ochre alleles (except $leu_{2.1}$, which is unaffected) responded to selection in the same direction, and both amber alleles responded in the other direction.

As stated above, a mechanism whereby a second suppressor reduces

Table 16a Liquid Growth Experiment 10 (Unselected lys^{+}_{6})

□ -AD

○ -LYS

■ -LEU

◆ -URA

△ -MET

▼ -TRY

● +ALL

Note change of scale on horizontal axis compared with all other graphs.

Fig. 16b Liquid Growth Experiment 10 (Selected $lys^{+}6$)

□ -AD

○ -LYS

■ -LEU

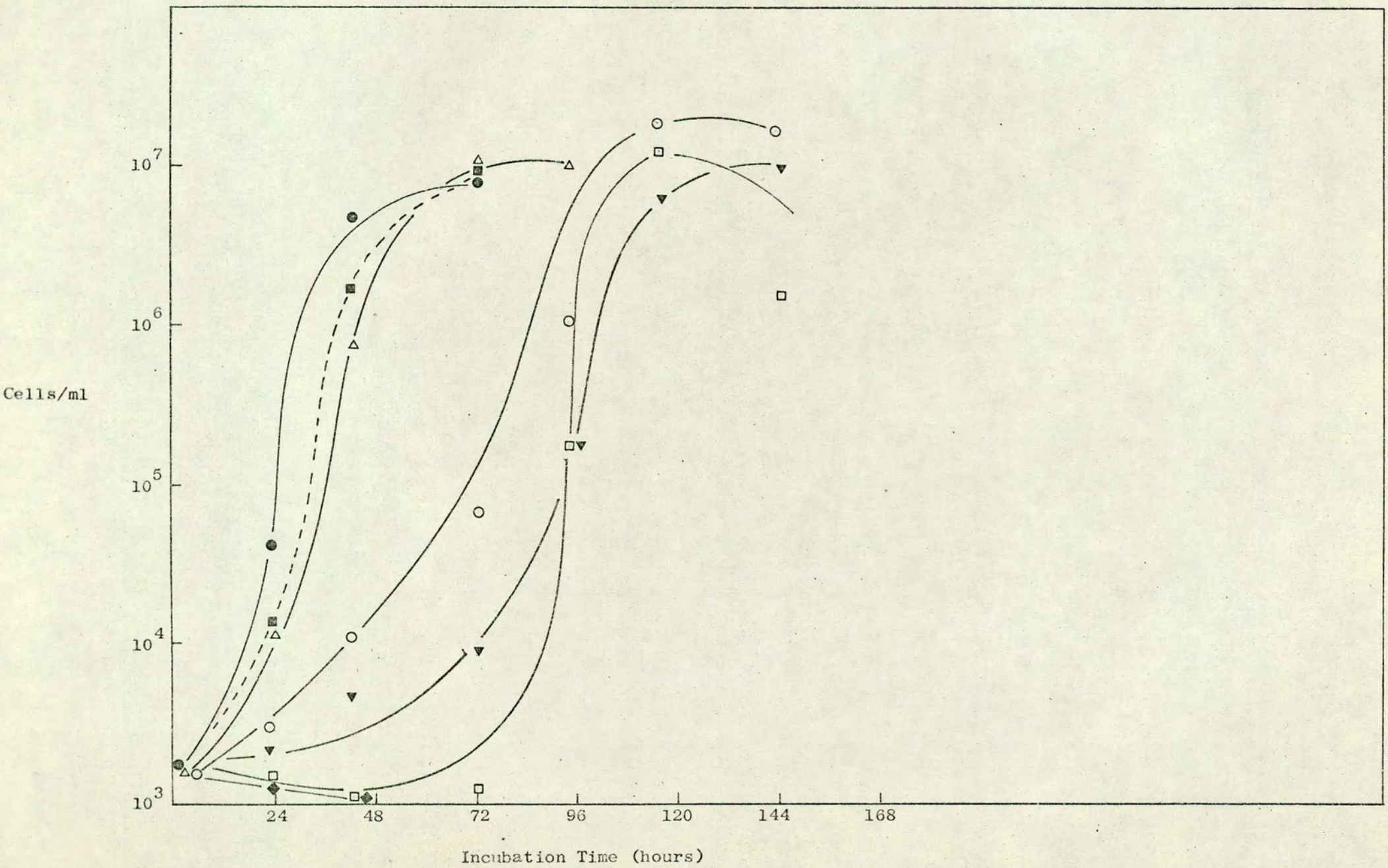
◆ -URA

△ -MET

▼ -TRY

● +ALL

Fig. 16b


10^7
10^6
10^5
10^4
10^3
Cells/ml
24
48
72
96
120
144
168
Incubation Time (hours)

the efficacy of the first is difficult to visualize, except perhaps if the former codes for a ribosomal component altered in such a way as to favour mistranslation of amber codons while at the same time favouring termination rather than suppression at ochre codons. More probably, a single suppressor, potentially able to translate both UAA and UAG, may be present, the response to selection involving the relative codon recognition specificity of the suppressor. Cytoplasmic conditions or an internal or external modifier gene may render UAA in unselected lys^+6 and UAG in selected lys^+6 the more readily suppressed nonsense codon. In view of the gradual enhancement of amber-suppressing ability concomitant with gradual loss of ochre-suppressing ability, the involvement of cytoplasmic state was considered the most likely of the above possibilities.

On any "modification" hypothesis, the different responses in -LEU and -URA can be explained as follows: growth in -URA takes place only in the unselected strain in which ochre suppression is more efficient than amber suppression; $ura_{4.1}$ may be less easily suppressed than other ochre alleles and hence be a more sensitive gauge for assessing levels of ochre suppression. Growth in -LEU is maximal in both unselected and selected lines; $leu_{2.1}$ may be very easily suppressed, allowing good growth even under conditions of inefficient ochre suppression.

In order to facilitate distinction between the various hypotheses, lys^+6 had to be outcrossed to produce a diploid homozygous for at least one ochre and one amber allele. Only then could segregation of ochre and amber suppressing ability be effectively monitored in

dissected asci. To this end, 2885-32B was crossed to 69/1/10, a haploid derivative of 69/1 having the genotype $ad_{2.1}$ $arg_{4.17}$ $lys_{1.1}$ $his_{5.2}$ $leu_{1.12}$ met^{+} try^{+}. The diploid was sporulated. Multiply-marked haploids were obtained from the 17 tetrads analysed. Ad^{-}, arg^{-}, lys^{-} and ura^{-} haploids were ochre mutants, while try^{-} and met^{-} were amber mutants. The two different ad^{-} mutants in the cross made determination of the exact ad^{-} genotype impossible without further analysis except in those cases where it could be deduced from the colour ($ad_{2.1}$ $ad_{5,7}^{+}$ haploids being pink) together with the segregation of adenine requirement.

Unselected $lys^{+}6$ was crossed to 4 multiply-marked haploids. Two of these haploids, 2885/3 and 2885/6, both of phenotype ad^{-} arg^{-} lys^{-} ura^{-} met^{-} try^{-}, crossed successfully and were then also crossed to selected $lys^{+}6$. Both crosses had to be made by mass mating, so single diploid colonies were not immediately obtained. An odd phenotype on PA characterized the mass mated crosses. The cells appeared diploid in that they were of a large, round shape with few buds present, but the few asci that could be seen (and some crosses appeared devoid of all asci) were of irregular shape and usually contained 2 or 3 spores only. Identification of diploid clones was therefore not always definite.

Presumed diploids of 2885/3 with $lys^{+}6$, both unselected and selected, were test-streaked on various omission media to test for dominance of suppressor activity. Results are shown in Table 11.

The unselected suppressor was fully dominant with respect to $lys_{1.1}$ suppression, but only partially so for $ura_{4.1}$ suppression.

Table 11 Growth Phenotypes of Diploids of Selected and Unselected lys^+6 crossed to 2885/3

Diploid Genotype : $\frac{ad_{5,7-101'}}{+}$ $\frac{+}{ad_{2.1}}$ $\frac{lys_{1.1}}{lys_{1.1}}$ $\frac{ura_{4.1}}{ura_{4.1}}$ $\frac{try_{1.1}}{try_{1.1}}$ $\frac{met_{8.1}}{met_{8.1}}$

\- : no growth

\+ : poor growth

++ : fair growth

+++ : good growth

Table 11

Diploid		-AD	-LYS	-URA	-TRY	-MET	+ALL
Unselected	1	+++	+++	-	-	-	+++
lys^+ 6 x 2885/3	2	+++	+++	+	-	-	+++
	3	+++	+++	++	-	-	+++
	4	+++	+++	++	-	-	+++
Selected	1	+++	-	-	-	++	+++
lys^+ 6 x 2885/3	2	+++	+	-	-	++	+++
	3	+++	-	-	-	++	+++
	4	+++	+	-	-	++	+++

(2885/3 was known to contain one or both of the ad^- ochre mutants $ad_{5,7-101}$ and $ad_{2.1}$. Subsequent tetrad analysis revealed its probable genotype as $ad_{5,7}^+$ $ad_{2.1}$. Since neither ad^- locus was homozygous, no conclusions regarding dominance at AD loci can be drawn from the results). No suppression of $met_{8.1}$ or $try_{1.1}$ was seen.

The suppressor in the selected line gave a different phenotype: partially dominant suppression of $met_{8.1}$ but no suppression of $try_{1.1}$ or $ura_{4.1}$, and partially dominant suppression of $lys_{1.1}$ was apparent.

In general, results in the diploid reflected those found in the haploid state, though ochre suppression appeared stronger and amber suppression weaker than in the selected haploid. Thus the effects of selection survived, at least partially, to the diploid stage. Selected suppressor activity resulting from impairment of function of a ribosomal protein is therefore unlikely, since such activity is expected to be recessive.

It was hoped that tetrad analysis might reveal the effects of meiosis on the selected suppressor activity. Diploid No. 3 of unselected lys^+6 and Diploid No. 1 of selected lys^+6 crossed with 2885/3 were sporulated. While the former sporulated well, the latter did so very poorly (as did selected 4c lines - see Section II c) ii) 1). Due to high spore inviability, only $^5/_{13}$ dissected asci of Diploid No. 3 and $^6/_{14}$ of Diploid No. 1 produced tetrads. All tetrads were test-streaked. Results are shown in Tables 12 and 13.

The segregations of lys^- and ura^- were expected to indicate the segregation of an ochre suppressor and those of met^- and try^- to

Table 12 Segregation Patterns in Diploid No. 3 of Unselected $lys^{+}6$ crossed to 2885/3

Diploid Genotype: $\frac{ad_{5,7-101'}}{+}\ \frac{+}{ad_{2.1}}\ \frac{+}{arg_{4.17}}\ \frac{lys_{1.1}}{lys_{1.1}}\ \frac{ura_{4.1}}{ura_{4.1}}\ \frac{try_{1.1}}{try_{1.1}}\ \frac{met_{8.1}}{met_{8.1}}$

\- : no growth

\+ : poor growth

++ : fair growth

+++ : good growth

Table 12

Tetrad		-AD	-ARG	-LYS	-URA	-MET	-TRY	+ALL
1	a	+++	+++	+++	++	-	-	+++
	b	+++	-	-	-	-	-	+++
	c	-	+++	-	-	-	-	+++
	d	+++	+++	+++	++	-	-	+++
2	a	+++	+++	+++	++	-	-	+++
	b	-	+++	-	-	-	-	+++
	c	+++	+++	+++	++	-	-	+++
	d	+++	-	-	-	-	-	+++
3	a	++	+++	+++	++	-	-	+++
	b	+++	+++	+++	++	-	-	+++
	c	-	-	-	-	-	-	+++
	d	-	+++	-	-	-	-	+++
4	a	+++	+++	+++	++	-	-	+++
	b	+++	-	-	-	-	-	+++
	c	+++	+++	+++	++	-	-	+++
	d	+++	+++	-	-	-	-	+++
5	a	+++	+++	+++	++	-	-	+++
	b	+++	-	-	-	-	-	+++
	c	+++	+++	+++	++	-	-	+++
	d	-	+++	-	-	-	-	+++

Table 13 Segregation Patterns in Diploid No. 1 of Selected $lys^{+}6$ crossed to 2885/3

Diploid Genotype: $\frac{ad_{5,7-101'}}{+}$ $\frac{+}{ad_{2.1}}$ $\frac{+}{arg_{4.17}}$ $\frac{lys_{1.1}}{lys_{1.1}}$ $\frac{ura_{4.1}}{ura_{4.1}}$ $\frac{try_{1.1}}{try_{1.1}}$ $\frac{met_{8.1}}{met_{8.1}}$

\- : no growth

\+ : poor growth

++ : fair growth

+++ : good growth

Table 13

Tetrad		-AD	-ARG	-LYS	-URA	-MET	-TRY	+ALL
1	a	-	-	-	-	-	-	+++
	b	+++	+++	+	-	+++	++	+++
	c	-	++	-	-	-	-	+++
	d	-	-	-	-	++	-	+++
2	a	-	+++	-	-	++	-	+++
	b	++	+++	+++	-	+++	++	+++
	c	+++	-	-	-	-	-	+++
	d	-	+++	-	-	-	-	+++
3	a	+++	+++	+	-	+++	++	+++
	b	-	-	-	-	-	-	+++
	c	+++	+++	+++	-	+++	++	+++
	d	-	-	-	-	-	-	+++
4	a	-	-	-	-	-	-	+++
	b	+	+++	-	-	+	+	+++
	c	-	-	-	-	+	-	+++
	d	-	++	-	-	-	-	+++
5	a	+++	+++	-	-	-	-	+++
	b	-	-	-	-	-	-	+++
	c	+	+++	+++	-	+++	++	+++
	d	-	-	-	-	-	-	+++
6	a	-	-	-	-	-	-	+++
	b	+	+++	+++	-	+++	++	+++
	c	++	+++	-	-	-	-	+++
	d	-	+++	-	-	-	-	+++

reflect that of an amber suppressor.

In Diploid No. 3, lys^- and ura^- segregated $2^+:2^-$ and met^- and try^- $0^+:4^-$ as expected for a heterozygous ochre-specific suppressor. Growth on -URA was weak. Segregation of arg^- was consistent with its being heterozygous in the diploid and suppressible by the ochre-specific suppressor.

Haploid 2885/3 was ad^-. The pattern of segregation of ad^- shown by Diploid No. 3 is only explicable if, in addition to the suppressor locus, both AD 5,7 and AD 2 loci were heterozygous in the diploid, and $ad_{2.1}$ is suppressible by the ochre suppressor. 2885/3 was deduced to be $ad_{5,7}^+$ $ad_{2.1}$. It was concluded that the unselected lys^+6 isolate contained an ochre-specific suppressor, presumably derived from a mutated tRNA gene.

Segregation in Diploid No. 1 was unusual. Met^- segregated $2^+:2^-$ in 4 tetrads and $1^+:3^-$ in the remaining 2. Try^- segregated $2^+:2^-$ only in one tetrad and $1^+:3^-$ in each of the other 5. In no case was a try^+ clone also met^-. A heterozygous amber suppressor could account for these segregation patterns if low penetrance of amber suppression, especially for $try_{1.1}$, is assumed.

Ura^- consistently segregated $0^+:4^-$, while lys^- segregated $0^+:4^-$, $1^+:3^-$ and $2^+:2^-$, indicating lower penetrance of ochre-suppressing ability than that found in haploids derived from Diploid No. 3 (made using unselected lys^+6). While fewer arg^+ and ad^+ spores were obtained from Diploid No. 1 than from Diploid No. 3, all lys^+ spores were also ad^+ and arg^+, this also being consistent with reduced expression of ochre suppression as proposed for the selected lys^+6

haploid. 25 tetrads from a mixed zygote culture gave similar results.

It was noted that growth on several media, especially that on -TRY and -LYS, was of unusual appearance, with discrete colonies of all sizes growing over low background growth. Such a phenotype was very similar to that previously found in some unselected suppressed $ad_{2.1}$ strains. This similarity could be construed as evidence for the comparability of the two situations, both perhaps being caused by suppression at low efficiency.

The segregation of amber and ochre alleles in Diploid No. 1 was consistent with the presence of a single ochre-amber suppressor of variable, and often low, penetrance. The original suppressor, present in Diploid No. 3, was ochre-specific. The selected amber-suppressing activity may have arisen either via an intra-genic modification of the suppressor or by selection of cytoplasmic conditions conducive to the translation of UAG by the suppressor. The facts that the selective effect was accumulated gradually and that tRNA-mediated amber-ochre suppressors have not been identified in yeast (Hawthorne and Leupold, 1974), argues in favour of the latter possibility.

It should be noted that, if cytoplasmic modifications are indeed involved, they are of sufficient stability both to survive in the absence of selection pressure and to pass through meiosis to allow full or partial suppressor activity in haploid progeny. However, it is unclear why, if selection caused a shift in cytoplasmic conditions affecting translation, such that recognition and translation of UAG is favoured at the expense of that of UAA, several spores of Diploid

No. 1 grew well on both -LYS and -MET, rather than well on one and poorly on the other.

The only conclusion that could be drawn from the above results was that the relative ability of a suppressor to translate UAA or UAG codons could be modified by selection of appropriate genic and/or cytoplasmic conditions. The gradual nature of the response to selection made some involvement of the cytoplasmic system in this effect plausible. Had time permitted further analysis, studies with tetrads from Diploid No. 1, involving detection of unexpressed suppressors and the conditions under which their ochre and/or amber suppressing ability could be expressed, may have thrown light on the situation. However, since sole involvement of cytoplasmic state in the effects of selection was not clear, this line of investigation was discontinued.

Selection Experiment 4

After incubation for approximately 12 days, a single colony appeared on the -LEU test-streak of isolate "try^{+}2". This isolate grew on -TRY plates after a single days incubation, but its phenotype regarding -MET growth was ambiguous, in that slight background growth only could be seen after 5 days of incubation. If this latter growth represented weak suppression, an amber-specific suppressor would be assumed to be present. However, such growth might have been the result of some other mechanism unrelated to suppression; if this were the case, a true try^{+} reversion would also be possible.

The single colony on -LEU was restreaked onto -LEU. Confluent growth was obtained. On subsequent streaking onto all other omission

media, results similar, but opposite in effect, to those in Selection Experiment 3 were obtained. While the unselected, control, line grew only on -TRY and +ALL, the selected line (from -LEU) grew well on -AD, -LYS, -LEU and -URA but not on -TRY or -MET and rather slowly on +ALL. As in Selection Experiment 3, selection for suppression of the alternative nonsense codon resulted in loss of ability to suppress the original nonsense codon. In this experiment, the change in specificity was from amber to ochre and was attained apparently in a single step; in Selection Experiment 3, the change was from predominantly ochre to predominantly amber suppression, and was attained after a lengthy selection procedure.

Deselection, and selection from the few colonies arising on the -MET streak of the selected line after 8 days, failed to restore the original phenotype.

In Liquid Growth Experiment 11, selected and unselected lines of $try^{+}2$ were grown in one flask each of every liquid omission medium. Growth curves shown in Fig. 17a,b reflect, in general, the results found on plates. It is noteworthy, however, that the slow growth of the selected line on +ALL and YEA plates was not shown in liquid +ALL medium. Growth of the selected line in -MET and -TRY was observed by microscopic examination of the cultures after approximately 200 hours. When plated on YEA, however, these cells failed to form colonies entirely or produced colonies, after prolonged incubation, which were too small to count. The reason for this is unknown.

In this case, outcrossing of the unselected and selected lines could yield no relevant information (as the number of suppressors

Fig. 17a Liquid Growth Experiment 11 (Unselected try$^{+}_{2}$)

□ -AD

○ -LYS

■ -LEU

◆ -URA

△ -MET

▼ -TRY

● +ALL

Fig. 17a

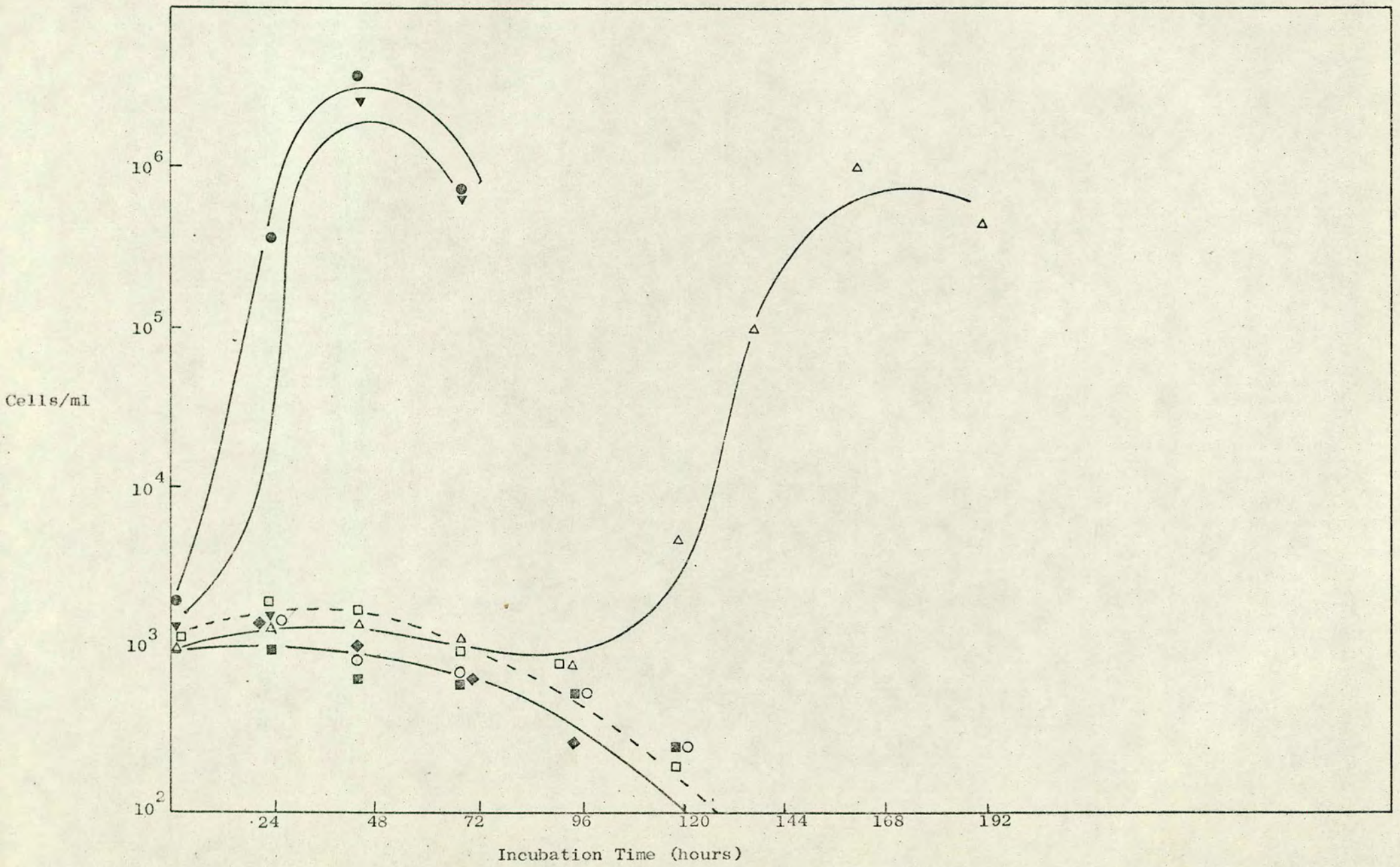

10^6
10^5
10^4
10^3
10^2
Cells/ml
24
48
72
96
120
144
168
192
Incubation Time (hours)

Fig. 17b Liquid Growth Experiment 11 (Selected $try^{+}2$)

□ -AD

○ -LYS

■ -LEU

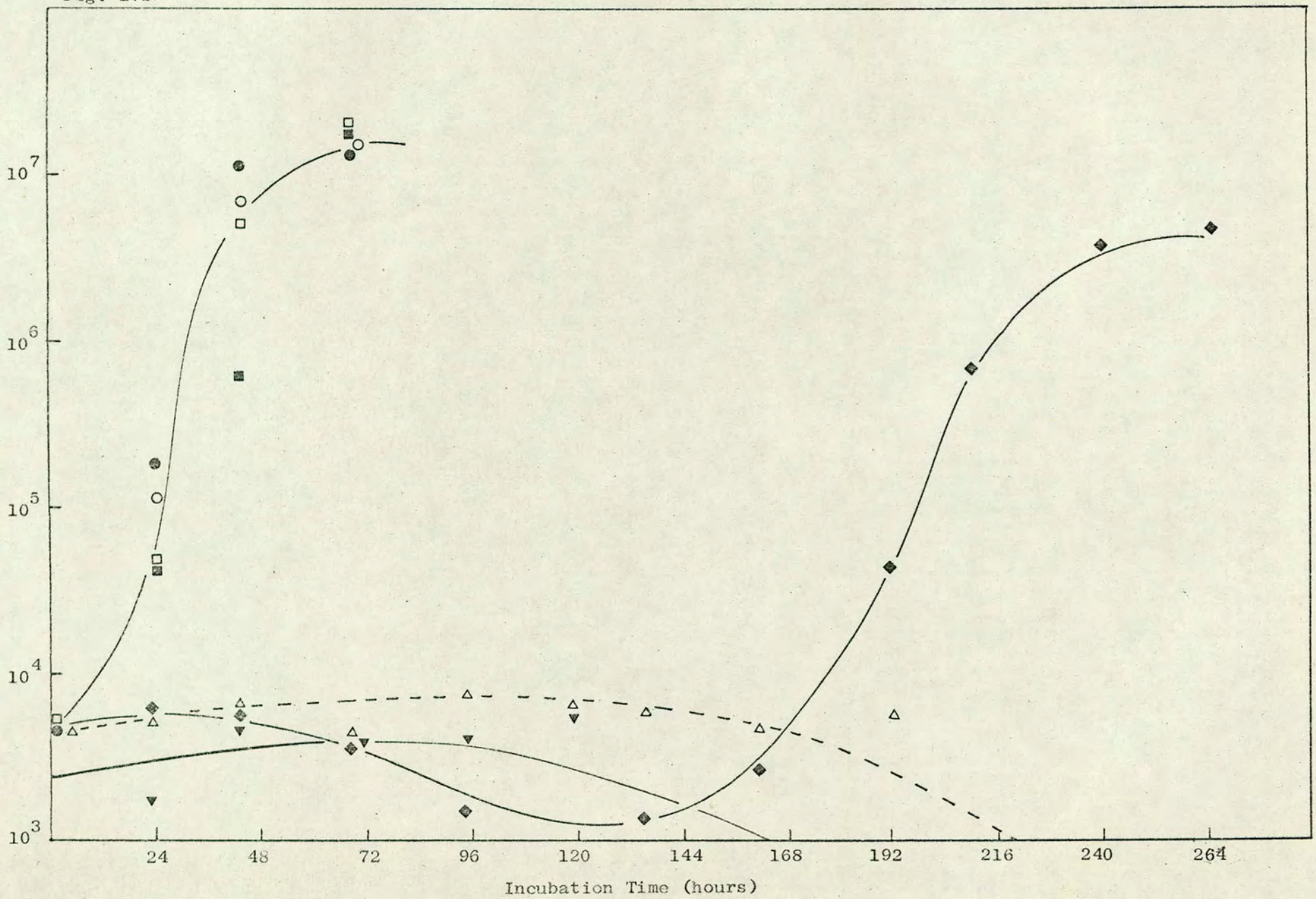
◆ -URA

△ -MET

▼ -TRY

● +ALL

Fig. 17b


10^7
10^6
10^5
10^4
10^3
Cells/ml
24
48
72
96
120
144
168
192
216
240
264
Incubation Time (hours)

present is not in doubt), so was not undertaken.

Such a complete difference in codon-recognition specificity in a single step from a single initial colony was thought more likely to be due to mutation than to selection of background cytoplasmic effects. A C to A base change in the presumed amber anticodon, CUA, giving AUA, followed by modification of the A in the first position of the anticodon rendering the anticodon ochre-specific (e.g. by deamination to give IUA) could account for the behaviour of the selected try^{+}2 isolate.

Summary of Selection Experiments

In none of the selection experimentswith suppressed 2885-32B isolates was the selection effect entirely attributable to a cytoplasmic change, although two isolates, leu^{+}4 (Experiment 2) and lys^{+}6 (Experiment 3) showed certain selection characteristics most easily interpreted in terms of such changes. Extension of these studies was thought likely to produce results of interest from the point of view of genic mutations able to modify suppressor expression, but as this was not the aim of this investigation, a different approach to the question of the role and potential influence of the cytoplasm in the suppression mechanism, was attempted.

If the leaky suppressed growth phenotype frequently observed on -URA were amenable to selection in the same way as were suppressed $ad_{2.1}$ strains, and both these selection effects had a common basis, then a suppressed $ad_{2.1}$ $ura_{4.1}$ strain, when selected for one growth phenotype, should display correlated selection for the other. Such a demonstration might provide evidence for a degree of cytoplasmic

control over the suppression system, this control being subject to manipulation to a certain extent by external environmental factors. With this in mind, the following set of experiments was carried out.

ii) Correlated Selection in Suppressed $ad_{2.1}$ $ura_{4.1}$ Haploid Strains

As previously described, tetrad analysis of a diploid made between 69/1/10 and 2885-32B yielded many multiply-marked haploid clones. Three asci segregated $0^+:4^-$ for adenine requirement and 2 pink:2 white for colour. In these three asci, therefore, the genotype at the AD 2 and AD 5,7 loci was known. Spontaneous suppressors were recovered in three $ad_{2.1}$ AD 5,7 $ura_{4.1}$ multiply-marked haploid isolates. Four different suppressed isolates of 5b, one of these three latter strains, were utilized in the following experiments. When originally scored, all four, arg^+1, lys^+2, lys^+3 and lys^+4, grew only at either end of -URA streaks. Arg^+1 and lys^+4 gave rise to only a single colony at one end of the -AD streak, while lys^+2 and lys^+3 showed intermittant growth along such streaks. Selection from -URA and -AD was carried out, using growth on +ALL as a control, as previously described (see Fig. 1). Selection was continued for 4 weeks, restreaking being carried out every 2-3 days.

Similar results were obtained for all 4 isolates. Selection from both -AD and -URA was successful, though the increase in growth rate obtained tended to vary between consecutive restreaks, and the total selective effect obtained was not as dramatic as seen previously in 69/1-derived strains. In general, confluent growth of final selected lines was obtained after approximately 3 days incubation.

A correlated effect of selection, in the form of improved growth ability on the omission medium not used in selection, was evident for all selected lines. It was interesting to note that, in the case of selection on -URA, the correlated increase in -AD growth ability frequently appeared to be greater than that in -URA growth ability. It was also noted that, on some occasions, growth rate of the -AD selected line on +ALL was lower than that of the unselected control line.

The above results can be most easily interpreted in terms of suppressor efficiency, although hypotheses based on modification of metabolic steps common to both biosynthetic pathways could also be proposed. If poor initial growth on -AD and -URA were a consequence of a suppression efficiency too low to give enzyme activities capable of sustaining wild-type growth rates on the appropriate omission media, selection for improved growth rate on one omission medium, if it occurs via increased suppression efficiency, might be expected also to lead to improved growth on the alternative omission medium. Since the increase in enzyme concentration required for a given increase in overall growth rate is unlikely to be identical for different enzymic steps, a given change in suppression efficiency resulting from selection is not expected to affect the growth rate on one medium to the same extent as that on the other. This could possibly account for those cases where, during selection on -URA, a slight improvement in growth on -URA was accompanied by a more obvious improvement in growth on -AD.

As noted previously, an increase in the efficiency of selection

may be detrimental to the cell, due to excessive translation of natural inter-cistronic chain-terminating codons. The reduced growth rate on +ALL noted above (and also in several selection experiments using 2885-32B) could be explained if selection does indeed increase the efficiency of suppression.

Relaxation of selection on +ALL re-introduced the original growth phenotype. It was concluded that the selection effects described are explicable in terms of a change in cytoplasmic state such that suppression efficiency is altered. If this is so, interpretation of results obtained using 69/1-derived strains in similar terms would appear to have some validity.

CHAPTER IV

DISCUSSION

The work described in this thesis was prompted by initial observations concerning the growth phenotype of suppressed $ad_{2.1}$ strains on -AD solid medium. The unexpectedly low and variable degree of suppression generally exhibited on -AD suggested the possible use of such strains in the study of factors governing suppression efficiency. With this in mind, various suppressed haploid and diploid strains were investigated. An apparent ability of all suppressed strains studied to "adapt" to -AD medium, both solid and liquid, was found. This adaptation took the form of improved growth rate on solid -AD medium and decreased lag period in liquid -AD medium. The magnitude of the adaptation response possible appeared to be governed to some extent by the suppressor genotype. The reversibility of adaptation phenomena on removal of the selection pressure exerted by -AD medium made physiological adaptation rather than mutant selection the more feasible basis for the effects described.

The biochemical basis of the hypothesized physiological adaptation was considered. Adaptation of either the adenine biosynthetic pathway or the suppression system itself was envisaged. Strain 2885-32B was examined in the hope of throwing light on the possibility of these two mechanisms.

Results obtained with suppressed isolates of 2885-32B did not provide unequivocal evidence for either possible mode of adaptation.

In one case (leu^+4 in Selection Experiment 2), acquisition of amber-suppressing ability in an ochre-suppressed strain was apparently not due to the occurrence of an amber suppressor gene, but may have been the result of an adaptation of the suppression system brought about by a change in cytoplasmic state imposed by the selective conditions. In a second case, selection in a suppressed $ad_{2.1}$ $ura_{4.1}$ haploid for growth ability on either -AD or -URA, resulted in a selection response on both media, again probably due to non-genetic factors. The correlated response to selection may be attributable either to an increase in suppressing ability (i.e. adaptation of the suppression system) or to modification of a metabolic step influencing both adenine and uracil biosynthesis.

The tentative conclusion was drawn that certain cellular adaptations, either physiological in nature or possibly involving distinct cytoplasmic elements whose qualitative and quantitative parameters are defined by the genotype, are possible and occur under conditions of physiological "stress" so that adaptation of the cell to new environmental conditions can take place. Such adaptations probably occur continuously since environmental conditions can rarely remain absolutely constant. Examples were cited in which environmental conditions produced obvious metabolic consequences; it therefore does not appear unreasonable to suppose that gradual adaptation of a cell population, perhaps requiring several generations to be completed, can occur. Such a phenomenon may not be widely documented because only in certain cases will such changes be phenotypically manifest and of such an extreme degree as to be easily measurable.

After conclusion of a large part of these studies, it was realized that the results obtained bore a strong resemblance to those of Kilkenny and Hinshelwood (1951) in their investigations of the utilization of galactose by yeast. These workers found that, while the absolute ability to utilize galactose depends on the presence of a normal Mendelian gene, different haploid isolates potentially capable of utilizing galactose display a varying growth capacity when first exposed to this substrate, this capacity increasing to an optimum on serial subculture in galactose medium. Specifically, an initial suspension of approximately 2-24 x 10^6 cells/ml of a strain able to utilize galactose grew in a liquid galactose medium after a lag of 2-5 days, with a mean generation time (m.g.t.) of about 1700 minutes. With each successive subculture a gradual decrease in lag period and m.g.t. was found until, at the 10th subculture, no lag period and a m.g.t. of about 170 minutes was obtained. Such adaptation or "training" to galactose was shown to be a response of the whole population of cells rather than due to the selection of a few mutant cells. An unrelated diploid strain grew in galactose medium at the optimum rate immediately, without any previous training. On sporulation, while absolute ability to utilize galactose appeared to segregate regularly, those haploid clones which could grow in galactose exhibited considerable variation in their ability to do so: the lag period at the first subculture varied from zero to 3000 minutes, while the m.g.t. varied from 155-650 minutes. All haploids, however, displayed an adaptive response in galactose medium, eventually growing after virtually no lag with

a m.g.t. of 170-180 minutes.

Certain similarities between the above results and those reported in the present investigation are immediately obvious. An su^+ gene may be thought of as analogous to the gene conferring galactose utilization ability, in that its presence is a prerequisite in $ad_{2.1}$ strains for potential ability to grow on -AD medium. However, strains carrying the required Mendelian gene which have not been exposed to the appropriate medium, show variation in their initial response to that medium; this is the case even when the same gene is known to be involved (e.g. when using haploid segregants of a heterozygous diploid). Background genetic and/or cytoplasmic effects clearly modify expression of the gene. The nature of either the nuclear gene or the background effects may sometimes render a strain apparently fully adapted on first exposure to the test medium (as for the second strain mentioned above and for some suppressed $ad_{2.1}$ haploid strains).

In both situations, serial transfer in test medium gradually leads to adaptation to that medium, this response being seen as an increased capacity for growth on or in such medium. The selection of nuclear mutants was discounted in both cases as the cause of the adaptation response.

Kilkenny and Hinshelwood (1951) conclude that, "Since auto-synthesis depends upon a complex interplay of nucleic acid and protein, and no doubt of other constituents", no gene can exist or function in isolation, so the "presence (of the gene for galactose utilization) only determines the extent of the Gal^+ character when

taken in relation to all the processes concerned in galactose metabolism". Furthermore, "optimal behaviour in a given biochemical reaction is only likely to be achieved by a gradual development of various configurations and proportions in the presence of the relevant substrate". They conclude that the phenomenon of adaptation is not incompatible with the Mendelian view of heredity; rather, it is only to be expected upon consideration of the interplay of the cell's constituent molecules in the production of the cell phenotype.

Similar conclusions have been reached concerning the results reported in this thesis: maximal expression on -AD of a given suppressor's activity in any particular $ad_{2.1}$ strain appeared to occur only after exposure of the strain to -AD medium for a certain period of time; some process of adaptation to -AD was inferred to be taking place during this exposure.

While adaptation to galactose utilization would seem likely to involve some aspect of the galactose metabolic pathway, the situation in the present case is not so obvious; adaptive modification of either the adenine biosynthetic pathway or the suppression mechanism seems possible.

A further example of physiological adaptation was provided by Drabble and Hinshelwood (1961) using the bacterium Bact. lactis aerogenes (Aerobacter aerogenes). In this case, adaptation to resistance to the drug streptomycin was involved. Development of full resistance to streptomycin appeared to involve primarily a "first-stage" mutation; expression of this mutation could be modified by exposure to various drug concentrations. For example, a

first-stage mutant selected as being resistant to 1 μg streptomycin/ml, required 5 passages in medium containing 1000 μg streptomycin/ml before it was able to grow with optimal efficiency at the higher concentration, a progressive decrease in m.g.t. (from 300 to 50-65 minutes) with each successive serial subculture being found. Moreover, while cells of the third 1000 μg streptomycin/ml subculture reverted to a m.g.t. of 300 minutes in 1000 μg streptomycin/ml after 15 passages in streptomycin-free medium, cells of the sixth subculture required 30 subcultures in drug-free medium before any reversion was detected (m.g.t. 87 minutes) and 77 subcultures to increase the m.g.t. to 250 minutes, and cells of the 19th subculture showed no reversion even after 82 drug-free passages. It therefore appeared that the stability of the adaptation to high drug concentrations increased with time of exposure to that concentration. This fact, and a reconstruction experiment involving growth of mixtures of trained and untrained cells, made the accumulation of mutants giving full streptomycin resistance unlikely.

Training of cells to 1000 μg streptomycin/ml was also carried out on solid medium by selecting according to colony diameter and constructing histograms of colony size distribution at each successive transfer. A gradual adaptation was indicated by an increase in average colony size. Whereas training in liquid medium appeared to be maximal after 5 or 6 subcultures, as judged by colony size distribution on spreading such cultures on plates containing 1000 μg streptomycin/ml, training on plates required at least 10 successive transfers before the maximal response was obtained.

The conclusion was reached that resistance to high streptomycin concentrations involved not only a mutation to streptomycin resistance, but also physiological adaptation of mutant cells to increase expression of that mutation to a maximum.

Again, several similarities between this bacterial situation and $ad_{2.1}$ suppression in yeast can be seen. The reduction in -AD lag period with increased adaptation of suppressed yeast strains may parallel the reduction in m.g.t. with number of transfers in drug medium of the bacterium. Furthermore, in both cases, the longer the exposure to the selective medium, the longer the exposure to non-selective medium required to negate the adaptation response. Also, in both cases, rather different results were obtained on exposure to solid rather than liquid medium.

Yet another example of adaptation concerns the utilization of D-arabinose as a carbon source by Bact. lactis aerogenes. Baskett and Hinshelwood (1951) presented many lines of evidence supporting adaptation of the whole bacterial population rather than selection of a few mutant cells, during the lag period preceding growth on initial exposure to D-arabinose solid or liquid medium. Induction by D-arabinose of a metabolic route potentially present in all cells of the population, and subject to repression by an alternative carbon source, glucose, was concluded to occur.

Phenotypic expression of certain female-sterile strains of Neurospora crassa is extremely variable and can be influenced by serial subculturing techniques (Fitzgerald, 1958, 1963). The phenotypic changes so produced display a limited inheritance. These

results have been interpreted as evidence for the interaction of the genotype with various cytoplasmic states or conditions in producing the spectrum of phenotypes. Different equilibrium states (either physiological or involving cytoplasmic elements) of a cellular system concerned with the phenotypic expression of a particular genotype may, in an epigenetic system, lead to changes in canalisation and thereby produce a range of phenotypes.

Jinks (1957) has described a continuous range in germination, growth rate, pigmentation and sexuality phenotypes of colonies arising from single asexual ascospores of Aspergillus glaucus. Selection for the cytoplasmic differences involved resulted in marked phenotypic changes, such changes being reversible by back selection and by heterokaryosis with an unselected line. The range and response to selection of the various phenotypic differences were attributed to variation in certain cytoplasmic elements, a change in the balance of which was responsible for selection responses.

Thus it appears that environmental stimulation of certain physiological mechanisms, requiring for completion lengthy though variable exposure of cells to that stimulus, is by no means a rare phenomenon.

While induction of metabolic pathways on exposure to certain nitrogen or carbon sources, or even adaptation of certain mutant cells to drugs such as streptomycin, present no conceptual difficulties, the process by which adaptation to -AD of suppressed $ad_{2.1}$ yeast strains could occur is not clear. Any proposed mechanism would have to be able to account for the following observations:-

1. The initial poor growth phenotype of suppressed strains on -AD, usually in the form of single isolated colonies growing on a streak of background cells, indicating growth of only a small proportion of cells (at least within a few days of incubation).

2. The ability of suppressed strains to be selected for increased growth rate on -AD plates, and the loss of this acquired capacity by growth on medium containing adenine.

3. In liquid -AD medium:

(i) Initial lag appears to be correlated with suppressor genotype.

(ii) Pregrowth in -AD liquid reduces lag on subsequent sub-inoculation into -AD medium.

(iii) Selection on -AD plates reduces lag on subsequent inoculation into -AD liquid.

(iv) Adaptation process can take up to at least 400 hours, during which time no cell division apparently takes place.

(v) Adapted cells (selected on -AD solid or in -AD liquid) can grow in +ALL liquid with normal growth rate, so the adaptation process has no significant lasting effect on general cell metabolism.

4. Adverse effects of adaptation are, however, manifest as reduced ability of diploids to sporulate and perhaps also of haploids to undergo diploidization. (The increased number of "giant" cells observed in selected lines may also reflect an imbalance in some aspect of metabolism apparently not reflected in an altered growth rate).

Although the molecular basis for the adaptive response obviously cannot be deduced from the data presented here, it may be worthwhile to speculate on the various possible biochemical mechanisms underlying

the response. Basically, two adaptation hypotheses must be considered, one in which the response to -AD is a result of modification of metabolic steps directly or indirectly affecting adenine biosynthesis, and the other in which the response derives from modification of some aspect of the suppression mechanism.

Let us consider first the former possibility. In this case, increased ability to grow on -AD is made possible despite a consistently low level of suppression and therefore a constant concentration of PAIC. In order that the same amount of PAIC formed by suppression be able to maintain a greater flux through the metabolic pathway(s) in which it acts in the adapted strain, the specific activity of this PAIC or the activity of other enzymes in the adenine biosynthetic pathway must increase. Since reaction conditions influence enzymic activity, the cellular milieu in which PAIC functions must affect, to some extent, its <u>in vivo</u> specific activity. Theoretically, therefore, PAIC specific activity may be modifiable by changes in cytoplasmic state resulting from prolonged exposure to -AD medium.

Since both adenine and uracil are nucleic acid bases, it is conceivable that they have a metabolic step in common, so a similar explanation for the correlated selection responses of ad^- and ura^- would not seem totally unreasonable. Several possible situations come to mind; however, in each case, in order that increased flux through the pathways be possible, it must be assumed that the enzymic step(s) modifiable by environmental changes is rate-determining, an inherently tenuous assumption in view of the fact that very few enzymic steps have this property (Kacser and Burns, 1973). Environ-

mental changes affecting, by chance, both mutant steps or a preceding non-mutant step in both biosynthetic pathways similarly, or affecting a single non-mutant step common to both pathways, could explain correlated responses of both ad^- and ura^-.

The possible ways in which the suppression mechanism could be modified to increase adenine production have already been enumerated. However, it may be useful to reconsider them now in more detail. It should be borne in mind that in all the following cases of environmental modification the enzymic steps involved must be assumed to be rate-determining.

Perhaps the most easily visualized adaptive mechanism is one which involves regulation of tRNA production. An increase in su^+tRNA synthesis, leading to increased suppression of ochre codons and hence increased PAIC concentration, may be brought about either by an increase in transcription of all tRNA genes or, perhaps less likely, in transcription of only the su^+tRNA locus. (It is assumed that the number of copies of the suppressor gene remains constant). While the mechanism by which this could be effected, and its connection with environmental conditions, is unknown, it does not seem unreasonable to suppose that transcription of tRNA genes is under some sort of cell regulatory control.

A second way to increase the effective level of su^+tRNA in PAIC formation would be either by increasing the proportion of total su^+tRNA reading the $ad_{2.1}$ ochre codon, or by increasing the suppression efficiency of su^+tRNA. It is not known whether compartmentalization of tRNA species exists; if it does, and can be modified by external conditions, such a modification could in principle, bring about

increased PAIC production. The reduced concentration of su^+ tRNA available for reading ochre codons of other suppressible mutations may still be sufficient to produce enough suppressed enzyme to maintain wild-type growth rates on appropriate omission media. However, correlated selection responses for ad^- and ura^- nonsense alleles are not easily explicable in terms of such a hypothesis.

The efficiency of suppression of any su^+ tRNA is determined by its ability to compete with chain termination factors. This ability is governed by numerous factors, both qualitative and quantitative (Krieg and Stent, 1968). The former include such features as the anticodon sequence (affecting strength of binding to the nonsense codon) and the reading context surrounding the nonsense codon (Salser, 1969; Salser *et al.*, 1969; Yahata *et al.*, 1970). These factors are clearly not subject to modification by environmental changes.

Factors affecting suppression efficiency which may have variable activity within a cell, and are therefore subject to modification, include: tRNA base-modifying enzymes whose activity can thereby affect tRNA function. For example, submodified forms of the *E. coli* amber suppressor su^+3 have reduced ability to bind to ribosomes and thus to suppress amber codons (Gefter and Russell, 1969). It has been suggested (Carbon and Fleck, 1974) that all *E. coli* nonsense suppressors may require for activity the modification of the base adjacent to the anticodon to N^6- (Δ^2-isopentenyl)-2-thiomethyl-adenylic acid. Since conditions of phage infection can apparently influence the extent of such base modifications (Gefter and Russell,

1969) and hence the efficiency of suppression, it could be argued that other growth conditions could similarly indirectly affect suppression efficiency. Such a situation might conceivably also exist in yeast.

The reaction between su^+tRNA and its cognate aminoacyl-tRNA synthetase might, in principle, be subject to environmental modification, increased su^+tRNA aminoacylation leading to greater overall suppression efficiency.

Other theoretically modifiable components of the translation mechanism are the protein release factors, decreased activity of which should lead to increased suppression efficiency.

Conformational changes of the ribosome can influence the ratio of translation to termination. For instance, strA and ram mutations of *E. coli*, which encode altered 30S ribosomal proteins, can affect the translation efficiency of su^+tRNAs (Strigini and Gorini, 1970; Biswas and Gorini, 1972). If such a conformational change could be brought about by an alteration in cellular environmental conditions consequent upon selection (analogous, for example, to the addition of streptomycin), modification of nonsense suppressor efficiency might result.

Increased transcription of a suppressible mutant locus may increase expression of a weak suppressor of that locus (Savic and Kanazir, 1975). Hence increased $ad_{2.1}$ suppression might result either from a general increase in transcription or from a specific increase in transcription of the AD 2 locus, this increase being a response to the environment of the adapting population.

Finally, since the functioning of any tRNA depends on its maintenance of a correct tertiary conformation, as evidenced by the isolation in E. coli and yeast of temperature-sensitive tRNA-mediated nonsense suppressors (Gallucci et al., 1970; Rasse-Messenguy and Fink, 1973), it is expected that a change in the conditions (e.g. ionic composition, pH) under which a suppressor functions may result in a change in its molecular configuration and thus affect one or more of the many reactions (with base-modifying enzymes, aminoacyl-tRNA synthetase, ribosomes, mRNA, etc.) in which it is involved. As stated previously, such a change induced by selection might hypothetically lead to modification of suppressor efficiency.

Environmental changes causing a different amino-acid to be loaded onto su^{+}tRNA, thereby increasing PAIC activity, appear highly unlikely; furthermore, such a hypothesis assumes that poor growth on -AD is due to insertion of a functionally unacceptable amino-acid. Since no suppressed 4c isolate, when first isolated, grew well on -AD, while a significant proportion of suppressors are expected to insert at the nonsense codon the amino-acid present in this position in wild-type PAIC, it seems unlikely that poor growth on -AD can be due to insertion of a functionally incompatible amino-acid.

The various modifiers of suppression efficiency described in the Introduction fail to provide a suitable basis for the interpretation of the results reported here. The reversibility of the modification of the -AD growth phenotype effectively rules out the involvement of any chromosomally-located modifier gene. The psi

system might, at first sight, appear to be a means by which suppressor efficiency could be varied on changing conditions external to the cell.

The identity of psi factors is as yet unknown. In fact, virtually all that is known about them is that they are cytoplasmically-inherited, depend for their maintenance and expression upon a nuclear gene, and influence the efficiency of nonsense suppressors. The location of the genetic information of psi determinants is unknown, as is whether psi^+ or psi^- is the natural state of yeast strains (both states being found in laboratory strains). It could be postulated that the selection response results from a change in the proportion of psi^- and psi^+ elements, both types being present in the unselected strain. If the psi^+ state causes increased suppression efficiency, unselected cells would contain a majority (or at least a sufficient number to be functionally dominant) of psi^- elements, and selected cells a majority of psi^+ elements. The selection response on -AD plates would reflect a change in equilibrium towards psi^+ factors within a population. On relaxation of selection pressure, the proportion of psi^+ elements would decrease, either via an active elimination of such elements or via dilution of psi^+ elements during vegetative reproduction. Both inter- and intra-strain differences in the unselected growth phenotype could perhaps be explicable in terms of variation in the proportion of psi^+ factors present. Adverse effects of selection, such as reduced sporulation ability, might be due to the interference with normal metabolic processes by the increased concentration of psi^+ factors.

The above hypothesis demands the assumption that, in the strains used, both psi^- and psi^+ elements are always present, neither selection nor deselection resulting in the total elimination of either type. (This follows from the observations that all suppressed strains tested could respond to selection, and that all selected strains reverted to the original unselected phenotype on relaxation of selection pressure). Furthermore, since virtually no suppressed strain was capable, when first isolated, of growing well on -AD, and since all relaxed strains reverted to the original phenotype, it must be assumed that, in all the strains studied, maintenance of a low frequency of psi^+ factors is favourable under "normal" growth conditions. It follows from this, and the adverse effects noted in selected lines, that an increased percentage of psi^+ factors is, in these strains, in some way detrimental to a suppressed cell when not under conditions of physiological stress. No such observations have been reported in other psi^+ or psi^- strains. It is unclear why the small proportion of psi^+ factors hypothesized to exist in unselected strains would be maintained at all; perhaps a certain number of psi^+ factors are required for some other function unrelated to modification of suppressor efficiency. If such a function exists, the adverse effects of selection, and the proposed reduction in number of psi^+ factors on removal of selection pressure, could be explained by the effect on this function of increased numbers of psi^+ factors.

The hypothesis is unable to provide an adequate explanation for the results obtained on outcrossing selected haploids. The results

of Cox (1965) involving crosses between psi^+ and psi^- strains could be explained if psi^+ elements were suppressive and were distributed to all four products of meiosis. However, the selected response in the strains used in this study did not appear to persist in the diploid or beyond meiosis when such strains were outcrossed; if an increase in number of psi^+ factors were responsible for the selection response, the apparent elimination of psi^+ in the diploid and haploid progeny is unexpected.

Other observations, such as the correlation between initial lag in -AD liquid with suppressor genotype, are similarly difficult to reconcile with a hypothesis based on the psi status of the cell. On the whole, therefore, modifications of the psi system are unlikely to explain the results described with suppressed $ad_{2.1}$ strains. However, the possibility of self-replicating cytoplasmic elements able to regulate chromosomal gene expression cannot be entirely eliminated. Such controlling elements might be located on some as yet unidentified species of cytoplasmic DNA.

In a recent publication, Young and Cox (1975) noted that $ad_{2.1}$ SUPQ5 psi^- strains, which are red and adenine-requiring (SUPQ5 will only suppress $ad_{2.1}$ in a psi^+ background), threw off numerous white papillae when grown on complete medium. Such a phenotype had also been observed for our red, suppressed 4c diploids on certain media, notably YEA. Indeed, such white papillae had been dissected during the early part of this work in the hope of demonstrating their identity or non-identity with the white colonies on -AD plates. Segregation at the ad^- locus was $2^+:2^-$; unfortunately, however,

due to aberrant segregation of all other markers displayed by 4c at this time, no conclusions regarding suppressor genotype could be drawn. Investigation of such white papillae was discontinued as probably being tangential to the main theme of this study, i.e. behaviour of suppressed strains on -AD medium.

Young and Cox (1975) concluded from analyses of white, ad^+ papillae that the majority of such papillae arose through a very high frequency of spontaneous mutation to weak recessive suppressors, SUPX, which could only suppress $ad_{2.1}$ in the presence of SUPQ5. It was suggested that SUPX, which by itself suppressed $try_{5.48}$, might be a special class of suppressor which could only be recovered in the genetic background $ad_{2.1}$ SUPQ5 psi^-.

Such conclusions regarding the nature of the white papillae are clearly at variance with those reached here regarding the nature of the white colonies found on -AD. This may simply be because the two phenomena are unrelated; alternatively, the psi^- strain investigated may have originally contained, by chance, both SUPQ5 and SUPX, variable penetrance of which gave rise to the observed phenotype (this accounting for the proposed high mutation rate to SUPX). The question obviously cannot be resolved without further experiments.

In summary, the conclusion is drawn that the expression of a suppressed $ad_{2.1}$ phenotype can be considerably modified by manipulation of environmental conditions. It is tentatively suggested that such modification is attributable to changes in certain aspects of the suppression mechanism rather than to changes in the activity

of the pseudo-wild-type enzyme produced by suppression. While no specific mechanisms for this can be advanced, some generalized characteristics of the system involved can be proposed on the basis of the observed properties of the suppressed strains.

On the premise that most cell populations display a continuous range of physiological states, most of this being non-inherited biological variation, the assumption is made that under certain culture conditions, a "heritable" physiological adaptation of cells can occur. Specifically, the expression at any one time of a potential $ad_{2.1}$ suppressor depends upon a number of variables, some or all of which are under the influence of certain environmental factors. Prolonged exposure of cells to a particular environment, in this case -AD, results in cytoplasmic modification allowing maximum expression of the suppressor and hence increased cell fitness in this environment.

A rough estimate of the efficiency of suppression can be estimated from the growth phenotype on or in -AD medium. Successive subculture on -AD plates caused a progressive increase in suppression efficiency in the population. This adaptation response was seen as a reduction in lag period in -AD liquid medium. Removal of selection pressure led to loss of the selective effect. Clearly, such cellular adaptation is "heritable" only when selective pressure is maintained.

The observations to be accounted for can be explained as follows;

1) The small proportion of cells of a suppressed isolate initially able to grow on -AD are those whose physiological state was, by chance,

capable of sustaining growth via suppression. That a smaller proportion of cells of diploid strains than of haploid strains initially grew on -AD may reflect either differences in their metabolic states or their decreased proportion of suppressor gene product per suppressible locus.

2) The various means by which selection might increase suppression efficiency, and thereby growth rate on -AD, have already been described. Any enforced cytoplasmic adaptation will be expected to be replaced by the metabolic state appropriate to YEA when the selective pressure is removed.

3) (i) Interpretation of results in liquid -AD medium presents some problems. That the initial -AD lag is apparently correlated with suppressor genotype suggests that the cytoplasmic state of the cell, which must be assumed to be different in haploids and diploids, in some way determines the nature of the suppressors which can be expressed during initial recovery of suppressors. Differences between the types of suppressor recovered in haploids and diploids must then be reflected in differing abilities of their gene products to participate in modification.

(ii) Pregrowth in -AD liquid reduces but does not always eliminate the lag on subsequent inoculation in -AD. If all growing cells were fully adapted to -AD, as appears likely from their wild-type growth rate, no requirement for further modification is expected on reinoculation into fresh -AD. The reason for the retention of some lag is obscure; possible, at the onset of stationary phase, culture conditions are such that the effective selection pressure

exerted is reduced and cells begin to revert to the original phenotype.

(iii) That selection on -AD plates reduces the subsequent lag in -AD liquid implies that similar adaptive mechanisms occur in both media.

(iv) Adaptation in liquid can apparently take many hours, and seems to occur in the absence of cell division. The length of the adaptation procedure needs no comment as we have no expectations for it. The failure of some strains to adapt may be a result of cell death occurring before adaptation can be completed.

(v) That adaptation apparently has no drastic, enduring effects on general cell metabolism, as judged by growth rate of selected strains in +ALL, might be because the adaptations involved are small in effect, being phenotypically manifest only under conditions of weak suppression.

4) The adverse effects which do occur may result from modification of certain cell constituents which also play a vital role in other processes such as sporulation.

The situation may be clarified by the use of biochemical techniques designed to detect qualitative and quantitative differences in su^+tRNA in unselected and selected strains. An additional approach, suggested by the work of Baskett and Hinshelwood (1951) might be the addition of a sterile filtrate from growing -AD cultures to an unadapted -AD culture to determine whether diffusible substances play a role in controlling the course of adaptation.

The use of known psi^- and psi^+ strains in selection experiments may help in determining whether such extra-chromosomal factors play

a part in selection responses. SUP05 in a psi$^-$ background, while never suppressing $ad_{2.1}$, is sometimes capable of suppressing $his_{5.2}$, $lys_{1.1}$ and can_{1-100}, although the latter growth is often limited (Young and Cox, 1975). It would be interesting to see whether selection for good growth is possible for these latter strains and, if so, whether the psi status shows a correlated change.

An indirect approach, useful only if positive results were obtained, might be an attempt to select for better growth of leaky missense auxotrophs. Demonstration of both selection and deselection of such mutants would make adaptation of PAIC activity seem more likely than at present.

REFERENCES

ALTMAN, S., BRENNER, S. and SMITH, J.D. (1971). Identification of an Ochre-suppressing Anticodon. J. Mol. Biol. 56, 195-197.

ANDOH, T. and GAREN, A. (1967). Fractionation of a Serine Transfer RNA containing Suppressor Activity. J. Mol. Biol. 24, 129-132.

BASKETT, A.C. and HINSHELWOOD, Sir C. (1951). The mechanism of the training of Bact. lactis aerogenes to D-arabinose. Proc. Roy. Soc. Series B. 139, 58-73.

BENZER, S. and CHAMPE, S.P. (1961). Ambivalent rII mutants of phage T4. Proc. Nat. Acad. Sci. US 47, 1025-1038.

BENZER, S. and CHAMPE, S.P. (1962). A change from Nonsense to Sense in the Genetic Code. Proc. Nat. Acad. Sci. US 48,1114-1121.

BISWAS, D.K. and GORINI, L. (1972). Restriction, De-restriction and Mistranslation in Missense Suppression. Ribosomal Discrimination of Transfer RNA's. J. Mol. Biol. 64, 119-134.

BOCK, R.M. (1967). Prediction of a Topaz Suppressor Ribonucleic Acid. J. Theoret. Biol. 16, 438-439.

von BORSTEL, R.C., PANUM, P.D. and STEINBERG, C.M. (1966). Nature of tr_{1-1} Super-suppressors in Saccharomyces. Science, 154, 430.

BRENNER, S., STRETTON, A.O.W. and KAPLAN, S. (1965). Genetic Code: The 'Nonsense' Triplets for Chain termination and their suppression. Nature, 206, 994-998.

BRUENN, J. and JACOBSON, K.B. (1972). New Species of Tyronine tRNA in Nonsense Suppressor Strains of Yeast. Biochim. Biophys. Acta 287, 68-76.

CAPECCHI, M.R. and GUSSIN, G.N. (1965). Suppression *in vitro*: Identification of a serine-sRNA as a "Nonsense" Suppressor. Science, 149, 417-422.

CARBON, J. and FLECK, E.W. (1974). Genetic Alteration of Structure and Function in Glycine Transfer RNA of *Escherichia coli*: Mechanism of Suppression of the Tryptophan Synthetase A78 Mutation. J. Mol. Biol. 85, 371-391.

CAVALLI-SFORZA, L.L. and LEDERBERG, J. (1953). In Symposium: Inhibitori di crescita and chemioterapia. Istituto Superiore de Sanità. Roma 1953. Genetics of resistance to bacterial inhibitors.

CHAN, T-S. and GAREN, A. (1969). Amino Acid Substitutions Resulting from Suppression of Nonsense Mutations. IV. Leucine Insertion by the su 6^+ Suppressor Gene. J. Mol. Biol. 45, 545-548.

CHAN, T-S. and GAREN, A. (1970). Amino Acid Substitutions Resulting from Suppression of Nonsense Mutations. V. Tryptophan Insertion by the su 9^+ Gene, a suppressor of the UGA Nonsense Triplet. J. Mol. Biol. 49, 231-234.

COX, B.S. (1965). A cytoplasmic suppressor of super-suppressor in yeast. Heredity, 20, 505-521.

COX, B.S. (1971). A Recessive Lethal Super-suppressor Mutation in Yeast and other Ψ phenomena. Heredity, 26, 211-232.

CRICK, F.H.C. (1966). Codon-Anticodon Pairing: The Wobble Hypothesis. J. Mol. Biol. 19, 548-555.

CRICK, F.H.C., BARNETT, L., BRENNER, S. and WATTS-TOBIN, R.J. (1961). General Nature of the Genetic Code for Proteins. Nature, 192, 1227-1232.

DRABBLE, W.T. and HINSHELWOOD, Sir C. (1961). Development of resistance to streptomycin by Bact. lactis aerogenes (Aerobacter aerogenes). I. The Role of Mutation and of Physiological Adaptation. Proc. Roy. Soc. Series B. 154, 449-462.

ENGELHARDT, D.L., WEBSTER, R.E., WILHELM, R.C. and ZINDER, N.D. (1965). In vitro studies on the mechanism of suppression of a Nonsense Mutation. Proc. Nat. Acad. Sci. US 54, 1791-1797.

EPHRUSSI, B. and HOTTINGUER, H. (1951). Cytoplasmic constituents of Heredity. On an Unstable Cell State in Yeast. Cold Spring Harbor Symp. Quant Biol. 16 75-84.

EPHRUSSI, B., HOTTINGUER, H. and ROMAN, H. (1955). Suppressiveness: A new factor in the Genetic Determinism of the Synthesis of Respiratory enzymes in Yeast. Proc. Nat. Acad. Sci US 41, 1065-1071.

FINK, G.R. (1966). A Cluster of Genes Controlling Three Enzymes in Histidine Biosynthesis in Saccharomyces cerevisiae. Genetics, 53 445-459.

FISHER, C.R. (1969). Enzymology of the Pigmented Adenine-requiring Mutants of Saccharomyces and Schizosaccharomyces. Biochem. Biophys. Res. Commun. 34, 306-310.

FITZGERALD, P.H. (1958). A case of Limitation of Phenotypic Variation. Nature, 181, 436.

FITZGERALD, P.H. (1963). Genetic and Epigenetic Factors Controlling Female Sterility in Neurospora crassa. Heredity, 18, 47-62.

GALLUCCI, E., PACCHETTI, G. and ZANGROSSI, S. (1970). Genetic Studies on Temperature Sensitive Nonsense Suppression. Mol. Gen. Genet. 106 362-370.

GAREN, A. (1968). Sense and Nonsense in the Genetic Code. Science, 160, 149-159.

GAREN, A. and SIDDIQI, O. (1962). Suppression of Mutations in the alkaline phosphatase structural cistron of E. coli. Proc. Nat. Acad. Sci. US, 48, 1121-1127.

GARTNER, T.K. and ORIAS, E. (1965). Pleiotrophic Effects of Suppressors of a lac - "Operator negative" Mutation in Escherichia coli. Proc. Nat. Acad. Sci. US, 53, 62-68.

GEFTER, M.L. and RUSSELL, R.L. (1969). Role of Modifications in Tyrosine Transfer RNA: A Modified Base Affecting Ribosome Binding. J. Mol. Biol. 39, 145-157.

GERLACH, W.L. (1975). Genetic Properties of some Amber-Ochre Super-suppressors in Saccharomyces cerevisiae. Mol. Gen. Genet. 138, 53-63.

GILMORE, R.A. (1967). Super-suppressors in Saccharomyces cerevisiae. Genetics, 56, 641-658.

GILMORE, R.A. and MORTIMER, R.K. (1966). Super-suppressor Mutations in Saccharomyces cerevisiae. J. Mol. Biol. 20, 307-311.

GILMORE, R.A., STEWART, J.W. and SHERMAN, F. (1968). Amino acid replacements resulting from Super-suppression of a Nonsense Mutant of Yeast. Biochim. Biophys. Acta, 161, 270-272.

GILMORE, R.A., STEWART, J.W. and SHERMAN, F. (1971). Amino Acid Replacements Resulting from Super-suppression of Nonsense Mutants of Iso-1-cytochrome c from Yeast. J. Mol. Biol. 61, 157-173.

GOODMAN, H.M., ABELSON, J., LANDY, A., BRENNER, S. and SMITH, J.D. (1968). Amber Suppression: a Nucleotide Change in the Anti-codon of a tyrosine Transfer RNA. Nature, 217, 1019-1024.

GOPINATHAN, K.P. and GAREN, A. (1970). A Leucyl-transfer RNA specified by the Amber Suppressor Gene Su 6^{+}. J. Mol. Biol. 47, 393-401.

GORINI, L. (1969). The Contrasting Role of str A and ram Gene Products in Ribosomal Functioning. Cold Spring Harbor Symp. Quant. Biol. 34, 101-109.

GORINI, L. (1970). Informational Suppression. Ann. Rev. Gen. 4, 107-134.

GORINI, L. and BECKWITH, J.R. (1966). Suppression. Ann. Rev. Micro. 20, 401-422.

GORMAN, J.A. and GORMAN, J. (1971). Genetic Analysis of a Gene Required for the Expression of allele-specific Missense Suppression in Saccharomyces cerevisiae. Genetics, 67, 337-352.

HARTMAN, P.E. and ROTH, J.R. (1973). Mechanisms of Suppression. Advances in Genetics, 17, 1-105.

HAWTHORNE, D.C. (1967). Genetic modifiers of super-suppressors in yeast. Genetics, 56, 563.

HAWTHORNE, D.C. (1969a). Identification of Nonsense Codons in Yeast. J. Mol. Biol. 43, 71-75.

HAWTHORNE, D.C. (1969b). The Selection of Nonsense Suppressors in Yeast. Mut. Res. 7, 187-197.

HAWTHORNE, D.C. and FRIIS, J. (1964). Osmotic-remedial mutants. A new classification for nutritional mutants in yeast. Genetics, 50, 829-839.

HAWTHORNE, D.C. and LEUPOLD, U. (1974). Suppressor Mutations in Yeast. Curr. Top. Microbiol. Immunol. 64, 1-47.

HAWTHORNE, D.C. and MORTIMER, R.K. (1963). Super-suppressors in Yeast. Genetics, 48, 617-620.

HAWTHORNE, D.C. and MORTIMER, R.K. (1968). Genetic Mapping of Nonsense Suppressors in Yeast. Genetics, 60, 735-742.

HIRSH, D. (1970). Tryptophan tRNA of Escherichia coli. Nature, 228, 57.

HIRSH, D. (1971). Tryptophan Transfer RNA as the UGA Suppressor. J. Mol. Biol. 58, 439-458.

HIRSH, D. and GOLD, L. (1971). Translation of the UGA Triplet in vitro by Tryptophan Transfer RNA's. J. Mol. Biol. 58, 459-468.

INGE-VECHTOMOV, S.G. (1965). Suppressors of Adenine requirement in Yeast. Genetika, 2, 22-26. Quoted in Hawthorne, D.C., Leupold, U. (1974). Suppressor Mutations in Yeast, Curr. Top. Microbiol. Immunol. 64, 1-47.

INGE-VECHTOMOV, S.G. (1966). Semidominant Super-suppressors in yeasts. Genetika, 7, 142. Quoted in Hawthorne, D.C. and Leupold, U. (1974). Suppressor Mutations in Yeast. Curr. Top. Microbiol. Immunol. 64, 1-47.

INGE-VECHTOMOV, S.G. (1967). Antisuppression. Genetika, 9, 176-178. Quoted in McCready, S.J. and Cox, B.S. (1973). Antisuppressors in Yeast. Mol. Gen. Genet. 124, 305-320.

JINKS, J.L. (1957). Selection for Cytoplasmic Differences. Proc. Roy. Soc. Series B. 146, 527-540.

KACSER, H. and BURNS, J.A. (1973). The Control of Flux. Symposia of the Society for Experimental Biology, 27, 65-104.

KAPLAN, S., STRETTON, A.O.W. and BRENNER, S. (1965). Amber Suppressors: Efficiency of Chain Propagation and Suppressor Specific Amino Acids. J. Mol. Biol. 14, 528-533.

KILKENNY, B.C. and HINSHELWOOD, Sir C. (1951). Adaptation and Mendelian segregation in the utilization of galactose by yeast. Proc. Roy. Soc. Series B. 139, 73-85.

KRIEG, R.H. and STENT, G.S. (1968). Bacterial Genetic Factors Controlling the Suppression of T4 Phage Amber Mutants. I. Suppression Patterns of a Collection of E. coli Strains. Mol. Gen. Genet. 103, 274-293.

LU, P. and RICH, A. (1971). The Nature of the Polypeptide Chain Termination Signal. J. Mol. Biol. 58, 513-531.

MAGNI, G.E., von BORSTEL, R.C. and STEINBERG, C.M. (1966). Super-suppressors as Addition-Deletion Mutations. J. Mol. Biol. 16, 568-570.

MAGNI, G.E. and PUGLISI, P.P. (1966). Mutagenesis of Super-Suppressors in Yeast. Cold Spring Harbor Symp. Quant. Biol. 31, 699-704.

MANNEY, T.R. (1964). Action of a Super-Suppressor in Yeast in relation to Allelic Mapping and Complementation. Genetics, 50, 109-121.

MANNEY, T.R. (1968). Evidence for chain termination by Super-suppressible Mutants in Yeast. Genetics, 60, 719-733.

McCREADY, S.J. and COX, B.S. (1973). Antisuppressors in Yeast. Mol. Gen. Genet. 124, 305-320.

MILLER, C.G. and ROTH, J.R. (1971). Recessive-lethal Nonsense Suppressors in Salmonella typhimurium. J. Mol. Biol. 59, 63-75.

MODEL, P., WEBSTER, R.E. and ZINDER, N.D. (1969). The UGA Codon in vitro: Chain Termination and Suppression. J. Mol. Biol. 43, 177-190.

NICHOLS, J.L. (1970). Nucleotide Sequence from the Polypeptide Chain Termination Region of the Coat Protein Cistron in Bacteriophage R17 RNA. Nature, 225, 147-151.

NIRENBERG, M., LEDER, P., BERNFIELD, M., BRIMACOMBE, R., TRUAN, J., ROTTMAN, F. and O'NEAL, C. (1965). RNA Codewords and Protein Synthesis. VII. On the General Nature of the RNA code. Proc. Nat. Acad. Sci. US, 53, 1161-1168.

NOTANI, G.W., ENGELHARDT, D.L. KONIGSBERG, W. and ZINDER, N.D. (1965). Suppression of a Coat Protein Mutant of the Bacteriophage f2. J. Mol. Biol. 12, 439-447.

OHLSSON, B.M., STRIGINI, P.F., BECKWITH, J.R. (1968). Allelic Amber and Ochre Suppressors. J. Mol. Biol. 36, 209-218.

OSBORN, M., PERSON, S., PHILLIPS, S. and FUNK, F. (1967). A Determination of Mutagen Specificity in Bacteria using Nonsense Mutants of Bacteriophage T4. J. Mol. Biol. 26, 437-447.

QUEIROZ, C. (1971). Mutation Induction in Yeast in Different Physiological States. Ph.D. thesis, University of Edinburgh.

RASSE-MESSENGUY, F. and FINK, G.R. (1973). Temperature-sensitive Nonsense Suppressors in Yeast. Genetics, 75, 459-464.

SALSER, W. (1969). The Influence of the Reading Context upon the Suppression of Nonsense Codons. Mol. Gen. Genet. 105, 125-130.

SALSER, W., FLUCK, M. and EPSTEIN, R. (1969). The Influence of the Reading Context upon the Suppression of Nonsense Codons, III. Cold Spring Harbor Symp. Quant. Biol. 34, 513-520.

SARABHAI, A.S., STRETTON, A.O.W., BRENNER, S. and BOLLE, A. (1964). Co-linearity of the gene with the Polypeptide Chain. Nature, 201, 13-17.

SAVIC, D.J. and KANAZIR, D.T. (1975). UV-induced Reversion Patterns of Constitutive and Repressed Salmonella Histidine auxotrophs. Mol. Gen. Genet. 137, 143-150.

SCHWEIZER, E., MacKECHNIE, C. and HALVORSON, H.O. (1969). The Redundancy of Ribosomal and Transfer RNA genes in Saccharomyces cerevisiae. J. Mol. Biol. 40, 261-277.

SIGNER, E.R., BECKWITH, J.R. and BRENNER, S. (1965). Mapping of Suppressor Loci in Escherichia coli. J. Mol. Biol. 14, 153-166.

SILVER, J.M. and EATON, N.R. (1969). Functional Blocks of the ad_1 and ad_2 Mutants of Saccharomyces cervisiae. Biochem. Biophys. Res. Commun. 34, 301-305.

SMITH, J.D., ABELSON, J.N., CLARK, B.F.C., GOODMAN, H.M. and BRENNER, S. (1966). Studies on Amber Suppressor tRNA. Cold Spring Harbor Symp. Quant. Biol. 31, 479-485.

SOIDLA, T.R. and INGE-VECHTOMOV, S.G. (1966). Influence of the genotype on allelic complementation and super-suppression in yeasts. Genetika, 9, 141. Quoted in Hawthorne, D.C. and Leupold, U. (1974). Suppressor Mutations in Yeast. Curr. Top. Microbiol. Immunol. 64, 1-47.

SÖLL, D. (1968). Studies on Polynucleotides. LXXXV. Partial Purification of an Amber Suppressor tRNA and Studies on in vitro Suppression. J. Mol. Biol. 34, 175-187.

SOLL, L. and BERG, P. (1969a). Recessive lethals: a new class of nonsense suppressors in Escherichia coli. Proc. Nat. Acad. Sci. US, 63, 392-399.

SOLL, L. and BERG, P. (1969b). Recessive lethal nonsense suppressor in Escherichia coli which inserts glutamine. Nature, 223, 1340-1342.

SOLL, D., OHTSUKA, E., JONES, D.S., LOHRMANN, R., HAYATSU, H., NISHIMURA, S. and KHORANA, H.G. (1965). Studies on Polynucleotides. XLIX. Stimulation of the Binding of aminoacyl-sRNA's to ribosomes by ribotrinucleotides and a survey of codon assignments for 20 amino acids. Proc. Nat. Acad. Sci. US, 54, 1378-1385.

STEWART, J.W. and SHERMAN, F. (1972). Demonstration of UAG as a Nonsense Codon in Bakers' Yeast by Amino-acid Replacements in Iso-1-cytochrome c. J. Mol. Biol. 68, 429-443.

STEWART, J.W. and SHERMAN, F. (1973). Confirmation of UAG as a Nonsense codon in Bakers' Yeast by Amino-acid Replacements of Glutamic Acid 71 in Iso-1-cytochrome c. J. Mol. Biol. 78, 169-184.

STEWART, J.W., SHERMAN, F., JACKSON, M., THOMAS, F.L.X and SHIPMAN, N. (1972). Demonstration of the UAA Ochre Codon in Bakers' Yeast by Amino-acid Replacements in Iso-1-cytochrome c. J. Mol. Biol. 68, 83-96.

STRETTON, A.O.W. and BRENNER, S. (1965). Molecular Consequences of the Amber Mutation and its Suppression. J. Mol. Biol. 12, 456-465.

STRIGINI, P. and GORINI, L. (1970). Ribosomal Mutations affecting Efficiency of Amber Suppression. J. Mol. Biol. 47, 517-530.

SUZUKI, T. and GAREN, A. (1969). Fragments of Alkaline Phosphatase from Nonsense Mutants. I. Isolation and Characterization of Fragments from Amber and Ochre Mutants. J. Mol. Biol. 45, 549-566.

TEVETHIA, M.J., BAPTIST, J.N. and MANDEL, M. (1974). Pleiotropic Effects of Suppressor Mutations in Bacillus subtilis. J. Bacteriol. 119, 961-975.

WEIGERT, M.G. and GAREN, A. (1965). Amino Acid Substitutions resulting from Suppression of Nonsense Mutations. I. Serine Insertion by the Su-1 Suppressor Gene. J. Mol. Biol. 12, 448-455.

WEIGERT, M.G., LANKA, E. and GAREN, A. (1965). Amino Acid Substitutions resulting from Suppression of Nonsense Mutations. II. Glutamine Insertion by the Su-2 Gene; Tyrosine Insertion by the Su-3 Gene. J. Mol. Biol. 14, 522-527.

WEIGERT, M.G., LANKA, E. and GAREN, A. (1967). Amino Acid Substitutions resulting from Suppression of Nonsense Mutations. III. Tyrosine Insertion by the Su-4 Gene. J. Mol. Biol. 23, 401-404.

WITKIN, E.M. (1963). One-step reversion to prototrophy in a selected group of multiauxotrophic substrains of E. coli. Genetics, 48, 916.

YAHATA, H., OCADA, Y. and TSUGITA, A. (1970). Adjacent Effect on Suppression Efficiency. II. Study on Ochre and Amber Mutants of T4 Phage Lysozyme. Mol. Gen. Genet. 106, 208-212.

YANIV, M., FOLK, W.R., BERG, P. and SOLL, L. (1974). A Single Mutational Modification of a Tryptophan-specific Transfer RNA Permits Aminoacylation by Glutamine and Translation of the Codon UAG. J. Mol. Biol. 86, 245-260.

YANOFSKY, C. and LAWRENCE, St. P. (1960). Gene Action. Ann. Rev. Microbiol. 14, 311-340.

YOSHIDA, M., TAKEISHI, K. and UKITA, T. (1970). Anticodon Structure of GAA-specific Glutamic Acid tRNA from Yeast. Biochem. Biophys. Res. Commun. 39, 852-857.

YOUNG, C.S.H. and COX, B.S. (1971). Extra chromosomal Elements in a Super-suppression System of Yeast. I. A nuclear gene controlling the inheritance of the extrachromosomal elements. Heredity, 26, 413-422.

YOUNG, C.S.H. and COX, B.S. (1972). Extrachromosomal elements in a Super-suppression system of yeast. II. Relations with other extrachromosomal elements. Heredity, 28, 189-199.

YOUNG, C.S.H. and COX, B.S. (1975). Extrachromosomal elements in a Super-suppression System of Yeast. III. Enhanced detection of weak suppressors in certain non-suppressed psi-strains. Heredity, 34, 83-92.

ZINDER, N.D., ENGELHARDT, D.L. and WEBSTER, R.E. (1966). Punctuation in the Genetic Code. Cold Spring Harbor Symp. Quant. Biol. 31, 251-256.

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ABSTRACT OF THESIS

Name of Candidate Janina Felicity Sliwowska

Address Department of Genetics, University of Edinburgh

Degree Ph.D. *Date* September, 1975

Title of Thesis Super-Suppression in Haploid and Diploid Yeast

Suppression of $ad_{2.1}$ haploids and homozygous diploids was observed to be far less effective than that of 4 other ochre nonsense alleles ($lys_{1.1}$, $arg_{4.17}$, $his_{5.2}$, $try_{5.48}$). The penetrance of the suppressed phenotype on adenineless medium (-AD) varied considerably and showed an approximate correlation with suppressor genotype. It was demonstrated that expression of suppressors on -AD plates could be considerably increased by a continual selection regimen. Loss of this improved growth ability by the growth of suppressed strains in a medium containing adenine, or by changes in their ploidy, suggested that increased suppressor expression resulted from physiological adaptation rather than from genetic change.

Growth patterns in liquid -AD were used to study some characteristics of the adaptation mechanism. Duration of the lag phase appeared to be correlated with initial ability to grow on -AD plates, and thus with the suppressor genotype. The slope of the exponential portion of the -AD growth curve was usually very similar to that in a comparable medium containing adenine (+ALL), suggesting that those cells capable of growth did so at the same rate in -AD as when adenine was not a limiting factor. However, although reinoculation of cultures grown in -AD into fresh -AD reduced the lag phase considerably, this phase was not usually eliminated; apparently, adaptation effects were easily lost.

The possibility that growth was attributable to selection of one or a small number of mutant cells was discounted on the grounds that not only did the -AD pregrowth effect of shortened lag period disappear after growth of such cultures in complete medium prior to reinoculation into fresh -AD, but also the length of the lag found in the first -AD culture was often such that any hypothesis based on selection of

Use other side if necessary.

mutant cells would also require to invoke an adaptation of such mutants.

Prior selection on -AD plates decreased the lag period in -AD liquid (or enabled growth to occur where none had before). It was deduced that at least some aspects of adaptation were common to responses to both selection media.

Selection experiments using suppressed isolates of strain 2885-32B, which contained a different set of nonsense alleles ($ad_{5,7-101}$, $lys_{1.1}$, $leu_{2.1}$, $ura_{4.1}$, $try_{1.1}$, $met_{8.1}$), revealed cases where selection for increased growth on one omission medium resulted in correlated growth responses on other omission media. The presence of additional suppressors was ruled out. Cytoplasmic changes affecting suppressor activity were invoked to explain these results.

Suppressed $ad_{2.1}$ $ura_{4.1}$ isolates similarly showed correlated responses to selection. Such responses were explicable in terms of modification either of inter-related biochemical pathways or of the suppression mechanism.

Possible models for the mechanism of adaptation were discussed. Assuming modification of suppressor efficiency underlies adaptive responses, such selection experiments were considered potentially useful in understanding various aspects of the suppression mechanism not readily analysed by classical genetic methodology.